JC03 Rec'd PCT/PTC 11 JAN 2002

FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**		ATTORNEY 'S DOCKET NUMBER 99/074 MED U.S. APPLICATION NO. (If known, see 37 CFR 1.5 10/030803
INTERNATIONAL APPLICATION NO. PCT/EP00/06214	INTERNATIONAL FILING DATE July 4, 2000	PRIORITY DATE CLAIMED July 15, 1999

TITLE OF INVENTION

"Cationic Block Copolymers"

APPLICANT(S) FOR DO/EO/US

Thomas Kissel; Holger Petersen: Dagmar Fischer; Klaus Kunath: Anke von Harpe

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☒ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☒ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

International Search Report; Return Receipt Post Card; cited references

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U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/030803	INTERNATIONAL APPLICATION NO. PCT/EP00/06214	ATTORNEY'S DOCKET NUMBER 99/074 MED

	CALCULATIONS	PTO USE ONLY
21. ☒ The following fees are submitted: **BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):** Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .... **$1040.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ....**$890.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO .... **$740.00** International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) .... **$710.00** International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	16 - 20 =	0	x **$18.00**	$ —	
Independent claims	2 - 3 =	0	x **$84.00**	$ —	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$	
				Amount to be refunded:	$
				charged:	$ 890.00

a. ☐ A check in the amount of $ ______ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ______ in the amount of $ ______ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. ______ . A duplicate copy of this sheet is enclosed.

d. ☒ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Klaus Schweitzer
ProPat, L.L.C.
2912 Crosby Road
Charlotte, North Carolina 28211-2815

K. Schweitzer
SIGNATURE

Klaus Schweitzer
NAME

See attached Limited Recognition Form
REGISTRATION NUMBER

10030803 .010402

10/030803
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE PATENT OPERATIONS

Art Unit:

Applicants: Thomas Kissel *et al.*

Application No.

Filed:

Title: Cationic Block Copolymers

Charlotte, North Carolina
January 11, 2002

Honorable Commissioner for Patents
Washington, DC 20231

Dear Sir:

PRELIMINARY AMENDMENT

Preliminary to Examination of the above-captioned application, kindly amend the application as follows:

In the Specification:

Please substitute the attached specification for the translation of PCT Application PCT/EP00/06214 filed on July 4, 2000, with the European Patent Office.

In the Claims:

Please substitute the attached claims for those included in the translation of PCT Application PCT/ EP00/06214 filed on July 4, 2000, with the European Patent Office.

REMARKS

Entry of the Amendment substituting the attached substitute specification and claims is respectfully requested by Applicants.

Respectfully submitted,

K. Schweitzer

Klaus Schweitzer
See attached Limited Recognition Under 37 CFR§10.9(b)
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Attorney's Docket No. 99/074 MED

Docket 99/074 MED

Final Version of PCT/EP00/06214 for U.S. prosecution:

"CATIONIC BLOCK COPOLYMERS"

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Holger PETERSEN
Dagmar FISCHER
Klaus KUNATH
Anke von HARPE

10/030803
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Background of the Invention

WO 98/59064 discloses PEI-PEG block copolymers and their use as vehicles for transporting nucleic acid into higher eukaryotic cells. The described copolymer was composed of branched PEI and linear PEG. PEI was PEGylated using methoxy-succinimidyl-propionate-PEG.

S. V. Vinogradov, T. K. Bronich and A. V. Kabanov (Bioconjugate Chem. 1998, 9, 805-812) describe the preparation of PEI-PEG and polyspermine-PEG block copolymers by using branched PEI and branched polyspermines through a coupling reaction with a monomethoxy-PEG activated with 1,1'-carbonyldiimidazole. The copolymers were used for complexation with oligonucleotides.

L. M. Bronstein, M. Antonietti et al. (Inorganica Chimica Acta 1998, 280, 348-354) describe PEI-PEG block copolymers and their preparation by coupling of branched PEI with monomethoxy-PEG which has a terminal acid chloride function, and the use thereof for preparing metal colloids.

V. Toncheva et al. (Biochimica et Biophysika Acta 1998, 138, 354-358) relates to block copolymers consisting of poly(L-lysine) and a plurality of hydrophilic polymers, such as PEG, dextran and poly(N-(2-hydroxypropyl)-methacrylamide, processes for their preparation and their use as vehicles for nucleic acid gene transfer.

These known block copolymers have the following three points in common:

1. The cationic polymer is equipped with side arms of a hydrophilic, nonionic polymer.

2. In all cases, for this purpose the reactive terminus of the hydrophilic, nonionic polymer was activated for the coupling reaction with the cationic polymer by a reagent which represents a linker between the blocks in the copolymer which is generated.

3. The hydrophilic, nonionic polymer was in all cases a linear polymer.

Summary of the Invention

Novel cationic block copolymers of the general formula I and II

(I) $A(-X-B)_n$

(II) $C(-Y-D)_m$

have been found, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 100 to 10 000 000 g/mol, preferably from 1000 to 100 000 g/mol and in particular from 5000 to 50 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 100 to 1 000 000 g/mol, preferably from 400 to 100 000 g/mol and in particular from 400 to 50 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures:

$-OC(O)NH(CH_2)_oNHC(O)NH-$ with o = 1 to 20, preferably 2 to 10, in particular 4 to 6,

-OC(O)NH(aryl)NHC(O)NH- with aryl = aromatic unit with, preferably, 6-14 C

atoms consisting of one or more aromatic nuclei which are connected together in fused or in polyphenylic form, preferably with one nucleus, in particular tolyl,

$-O(CH_2)_pC(O)NH-$ with p = 1 to 10, preferably 1 to 3, in particular 1,

$-OCH_2CH(OH)CH_2NH-$,

$-OC(O)NH-$, or

$-O(CH_2)_qNH-$ with q = 1 to 20, preferably 1 to 6, in particular 1 to 3;

n is an integer from
1) 1 to 200, preferably
2) 1 to 50,
3) 1 to 12,
4) 1 to 8 or, particularly preferably,
5) 2 to 8;

C is a linear or branched PEI with a molecular weight of from 100 to 1 000 000 g/mol, preferably from 400 to 100 000 g/mol, and in particular from 400 to 50 000 g/mol;

D is a residue of a polyethylene glycol which is linked via O of the formula

$-(CH_2CH_2O)_{n'}-R^1$

in which n' is from 3 to 25 000, preferably from 10 to 5000 and in particular from 10 to 1000, and R^1 is hydrogen, an aliphatic radical such as (C_1-C_6)-alkyl (methyl, ethyl, tert-butyl and the like) or another OH-protective group such as acyl (e.g. optionally substituted benzoxycarbonyl), optionally substituted benzyl, picolyl, or a cellular ligand in

order to bring about specific uptake of a nucleic acid-copolymer complex through binding to cell surface proteins, in particular receptors;

Y is a direct linkage of blocks C and D or a linker with the following structures:

$-NHC(O)NH(CH_2)_sNHC(O)O-$ with s = 1 to 20, preferably 2 to 10, in particular 4 to 6,

-NHC(O)NH(aryl)NHC(O)O- with aryl = aromatic unit with, preferably, 6 to 14 C atoms consisting of one or more aromatic nuclei which are connected together in fused or in polyphenylic form, preferably with one nucleus, in particular tolyl,

$-NH(CH_2)_tC(O)O-$ with t = 2 to 10, preferably 2 to 3, in particular 2,

$-NHCH_2CH(OH)CH_2O-$, or

$-NH(CH_2)_uO-$ with u = 1 to 20, preferably 1 to 6, in particular 1 to 3;

and

m is an integer from 1) 1 to 200, preferably 2) 1 to 100, in particular 3) 1 to 50.

Detailed Description of the Invention

The cationic block copolymers of the invention differ from the known block

copolymers in at least one of the following three features:

1. A hydrophilic, nonionic polymer is equipped with side arms of a cationic polymer.
2. The linkers differ from those of known block copolymers.
3. They have a branched hydrophilic, nonionic polymer.

A preferably means linear or branched polymers which are composed of carbon and oxygen and which may, where appropriate, also comprise cyclic, star or dendritic structures, such as, for example, residues of linear PEG, multi-arm branched PEG, star PEG, polysaccharides including cyclodextrins, PVA, arborols (dendrimers with terminal hydroxyl groups), but preferably linear and multi-arm branched and star PEGs. The latter are commercially available inter alia from Aldrich, Fluka, Sigma and Shearwater.

B and C mean linear or branched polyethyleneimine residues which have the formula III

(III) $-[CH_2CH_2N^{(z+)}(R^2)_x]_y-H[A^-]_w$

in which R^2 is identical or different radicals and is hydrogen or a radical of the formula IV

(IV) $-[CH_2CH_2N^{(z'+)}(R^3)_{x'}]_{y'}-H[A^-]_{w'}$

R^3 is identical or different radicals which (recursively) are defined as R^2,

A^- are an equivalent of a suitable, preferably inorganic anion such as OH^-, Cl^-, Br^- and the like,

x and x' are identical or different and are 1 or 2,

y and y' are identical or different and are integers which are chosen so that the radicals B and C have a constituent molecular weight of from 100 to 1 000 000 g/mol, preferably from 400 to 100 000 g/mol and in particular from 400 to 50 000 g/mol, it also being possible for y' to be 0,

z and z' are identical or different, z = x-1 and z' = x'-1,

and

w and w' are identical or different integers which are chosen so as to balance the positive charges in the radicals of the formulae III and IV.

The polyethyleneimines can be prepared in a manner known per se or are commercially available under the BASF brand name Lupasol® or under the name polyethyleneimine or ethyleneimine polymer in various molecular weights of from 400 to 2 000 000 g/mol (from Aldrich, Sigma, Fluka or directly from BASF). Preference is given to polyethyleneimines with a molecular weight of from 400 to 2000 g/mol for B and to polyethyleneimines with a molecular weight of from 400 to 800 000 g/mol, particularly preferably from 400 to 25 000 g/mol, for C.

The groups described under D are residues of polyethylene glycols which are protected on one terminus by a radical R^1 such as, for example, methyl or another suitable protective group. However, R^1 may also be a group which performs a specific or nonspecific biological function, in particular a ligand for interactions with receptors for target cell-specific uptake of a block copolymer-active substance complex into higher eukaryotic cells and the cell nucleus

thereof, where the active substance is preferably an oligonucleotide or a gene (gene targeting). R^1 can thus also be a ligand for a specific interaction and uptake into target organ tissue or cells, for example proteins, in particular antibodies or antibody fragments such as Fab, $F(ab)_2$, scFv,
cytokines or lymphokines, such as interleukins (IL-2 to x), interferon GM-CSF,
growth factors such as EGF, PDGF, FGF, EPO,
integrins such as ICAM, VCAM or
glycoproteins such as lectins or glycosilated proteins (see above) or
lipoproteins such as LDL, HDL or
transporter proteins such as transferrin or
peptides such as LH-RH, calcitonin, oxytocin, insulin, somatostatin, IGF, RGD or
carbohydrates such as galactose, mannose, glucose, lactose or
hormones such as steroids, THR or
vitamins such as B_{12}, folic acid.

The invention also relates to processes for preparing compounds of the formula I, which comprise

a) reacting compounds of the general formula V

(V) $A\text{-}(OH)_n$ with A and n = as in formula I

with diisocyanate, preferably hexamethylene diisocyanate, and reacting the compound resulting therefrom with polyethyleneimines with the general formulae III and IV, or

b) adding compounds of the general formula VI

(VI) $A\text{-}(NH_2)_n$ (with A and n = as defined in formula I)

to the reaction mixture for the polymerization of ethyleneimine before

the start of the polymerization or not until the polymerization is in progress, or

c) employing compounds of the general formula VII

(VII) $A\text{-}(OS(O)_2R^4)_n$ with A as in formula I and R^4 = aliphatic or aromatic radical, preferably p-tolyl, fluoride, trifluoromethyl or methyl,

as macroinitiator for the polymerization of ethyleneimine.

Compounds of the formula VI are commercially available in various molecular weights, for example from Shearwater.

Compounds of the general formula VII are obtained by reacting compounds of the general formula V with compounds of the general formula VIII

(VIII) $Cl\text{-}S(O)_2R^4$ (R^4 as defined above).

The invention further relates to processes for preparing compounds of the formula II, which comprise

d) initially reacting compounds of the general formula IX

(IX) D-OH (with D as defined in formula II)

with diisocyanate, preferably hexamethylene diisocyanate, and subsequently reacting the resulting compound with linear or branched polyethyleneimine. Protective groups introduced where appropriate to protect OH groups can be eliminated in a manner known per se (see, for example, Büllesbach, Kontakte (Merck) 1/1980, pp. 23 et seq.).

The process described under a) is preferably carried out in such a way that

a 4- to 20-fold excess of diisocyanate, preferably hexamethylene diisocyanate, is employed per terminal hydroxyl group of the polymer block A. The reaction is carried out in chloroform at temperatures from room temperature to the boiling point of the solvent, but preferably at the boiling point of the solvent. The chosen reaction time is between 2 and 24 hours, but preferably 4 hours. The polymer concentration in the reaction mixture is between 10 g/l and 500 g/l, preferably 100 g/l. The product is isolated by removing the solvent under reduced pressure and removing the excess diisocyanate by repeated extraction with petroleum ether (boiling range: 40-60°C). This intermediate is reacted with a 3- to 10-fold excess of PEI macromolecules per terminal hydroxyl group of the starting compound. The reaction is carried out in chloroform at temperatures from room temperature to the boiling point of the solvent, but preferably at the boiling point of the solvent. The chosen reaction time is between 6 and 72 hours, but preferably 12 hours. The polymer concentrations, both those of the PEI and those of the nonionic hydrophilic polymer which has been activated with hexamethylene diisocyanate, in the reaction mixture are between 10 g/l and 500 g/l, preferably between 30-200 g/l. The product is isolated by precipitating the polymer in a 10-30-fold volumetric excess of diethyl ether. Excess PEI can be removed from the block copolymer by repeated reprecipitation with ethanol and diethyl ether as solvent.

The process described under b) is carried out by mixing the ethyleneimine and the amino-terminated hydrophilic nonionic polymer in water in a concentration of from 10 g/l to 500 g/l in each case. The molar ratio of the two components is between 1:10 to 1:10 000. The ethyleneimine polymerization is then initiated by adding a suitable catalyst, for example hydrochloric acid, and the mixture is brought to a temperature of 40-100°C. The copolymer is generated by a chain termination reaction. The block copolymer is repeatedly reprecipitated with the aid of suitable solvents, for example ethanol and diethyl ether, and/or by pressure filtration, to remove PEI homopolymer which is a possible by-product. One variation of this

preparation process comprises adding the amino-terminated hydrophilic nonionic polymer to the hot polymerization mixture only after a certain reaction time of from 30 minutes up to 72 hours.

The process described under c) is carried out by reacting the terminal hydroxyl group(s) of the polymer block A with a sulfonyl chloride of the general formula VIII, but especially with toluenesulfonyl chloride (tosyl chloride). This reaction is carried out in aqueous and/or polar organic solvent, preferably in a water/tetrahydrofuran mixture, at temperatures of from -10°C to the boiling point of the solvent, preferably at temperatures of from -10°C to the boiling point of the solvent, preferably at temperatures of from 0°C to 25°C, and (if necessary) in the presence of catalysts such as, for example, triethylamine or sodium hydroxide. The product is isolated by removing the solvent under reduced pressure. This polymer is subsequently used as macroinitiator for the ethyleneimine polymerization. For this purpose, the product with the general formula VII is reacted with ethyleneimine in aqueous or polar organic solvent at temperatures of from 0°C to the boiling point of the solvent. The molar ratio of the two components is between 1:10 to 1:10 000. No by-product is formed in this reaction. The final product can be isolated by precipitating the polymer in a suitable solvent such as, for example, diethyl ether. The process described under d) is preferably carried out by reacting a compound of the general formula IX with a small excess, preferably a 2- to 10-fold excess, of diisocyanate, preferably hexamethylene diisocyanate. The reaction is carried out in chloroform at temperatures of from 20°C to the boiling point of the solvent, but preferably at the boiling point of the solvent. The chosen reaction time is between 2 and 24 hours, but preferably 10 to 14 hours. The polymer concentration in the reaction mixture is between 10 g/l and 500 g/l, preferably 30 to 150 g/l. The product is isolated by removing the solvent under reduced pressure and removing the excess diisocyanate by repeated extraction with petroleum ether (boiling range: 40-60°C). This intermediate is reacted with PEI macromolecules in a molar ratio of from 1:1 to 100:1. The reaction is carried out in chloroform and, if necessary, with

addition of dimethylformamide at temperatures from room temperature to the boiling point of the solvent, but preferably at 60-70°C. The chosen reaction time is between 6 and 72 hours, but preferably 12 hours. The polymer concentrations, both those of the PEI and those of the nonionic hydrophilic polymer activated with hexamethylene diisocyanate, in the reaction mixture are between 10 g/l and 500 g/l, preferably between 30-200 g/l. The product is isolated by precipitating the polymer in a 10-30-fold volumetric excess of diethyl ether.

Compared with PEI, the novel compounds have the following properties:

The block copolymers have a lower toxicity than PEI homopolymers in cytotoxicity tests and remain longer in the blood circulation (see "Biological Examples" section).

The block copolymers are more or less, depending on the structure, surface-active substances which can be used as surfactants.

In addition, the block copolymers can also be used

- in adhesive and coating systems as additive
- as fixing agents to improve paper strength
- as primers for polymer composite systems such as, for example, multilayer packaging sheets
- for modifying plastics (improving the dyeability, paintability, barrier effect)
- for fixing reactive dyes on cotton
- as coagulant and dispersant for fine suspended particles in industrial waste waters
- for binding heavy metal salts
- for dispersing organic and inorganic pigments
- as addition in ceramic and cement components
- for a wide variety of functions in skin and hair cosmetics and in the

dental sector

- for immobilizing medicinal active substances or bioactive compounds on surfaces
- for filtering endotoxins and pathogens out of blood plasma
- for penetration through mucous membranes.

In addition, in aqueous systems the block copolymers form complexes with polynucleic acids such as DNA and RNA, including ribozymes. This property makes them suitable as vehicles or vectors for gene transfer (penetration through cell membranes and translocation into the cell nucleus). They can therefore be used in transfection experiments, in gene therapy and diagnosis (see "Biological Examples" section).

The following examples serve to illustrate the invention without intending to restrict it thereto.

Chemical Examples

Example 1:

Preparation of a $PEI(PEG)_n$ block copolymer

Activation of mPEG-550

10 ml of chloroform are introduced into a 100 ml round-bottomed flask with magnetic stirring bar, reflux condenser and drying tube on top, and 7 ml of hexamethylene diisocyanate (HMDI) (43.64 mmol, 8 eq.) are added. 3 g of polyethylene oxide monomethyl ether (mPEG, M_n = 550 g/mol) (5.45 mmol, 1 eq.) are dissolved in 40 ml of chloroform. This solution is then slowly added dropwise to the stirred HMDI solution. The mixture is heated under reflux for 12 hours. The solvent is then removed under reduced pressure, and the excess HMDI is extracted with petroleum ether (40-60) (5x50 ml). The product

is obtained as a colorless mobile oil in virtually quantitative yield (3.8 g, 97%).

Preparation of a PEI-graft-PEG block copolymer

1.74 g of bPEI (M_w = 25 kDa, M_n = 10 kDa, 0.1736 mmol, 1 eq.) are weighed into a 100 ml round-bottomed flask with magnetic stirring bar, reflux condenser and drying tube on top, and dissolved in 40 ml of dimethylformamide (DMF). 2.5 g of the HMDI-activated mPEG (M_n = 720 Da, 3.47 mmol) are dissolved in 10 ml of chloroform, and this solution is slowly added dropwise to the stirred PEI solution. The mixture is heated at 60-70°C for 12 hours. The mixture is then added dropwise to 500 ml of diethyl ether. After two hours, a viscous yellowish oil has deposited. The cloudy supernatant is discarded, and the oil is dissolved in 30 ml of ethanol. The solution is again added dropwise to 500 ml of diethyl ether, and the oil which has again separated out is isolated by decantation. The product is dissolved in ethanol for filtration, and the solvent is removed in a vacuum oven at 50°C. 2.8 g of a yellowish viscous to resinous oil are obtained (yield: 45%).

The polymers were characterized by ^1H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 1. They are representative of the other examples, for which similar data were obtained.

^1H NMR (500 MHz, $CDCl_3$): δ/ppm = 1.17 (isocyanate CH_2), 1.26 (isocyanate CH_2), 2.30-2.72 (ethyleneimine CH_2), 2.96 (isocyanate CH_2), 3.15 (isocyanate CH_2), 3.49 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm 14.3 (isocyanate CH_2), 26.2 (isocyanate CH_2), 29.6 (isocyanate CH_2), 36.2 (isocyanate CH_2), 37.5 (ethyleneimine CH_2), 39.1 (ethyleneimine CH_2), 41.1 (isocyanate CH_2), 47.2 (ethyleneimine CH_2), 48.9 (ethyleneimine CH_2), 52.8 (ethyleneimine CH_2), 54.1 (ethyleneimine CH_2), 58.7 (isocyanate CH_2), 69.3 and 70.2 and 71.6 (ethylene

glycol CH_2), 156.2 (-NHC(O)O-), 161.7 (-NHC(O)NH-).

GPC (aminoethyl methacrylate gel, 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 8800, M_w = 1 640 000, M_p = 85 000, PD = 19.6, monomodal.
Comparison with blend of PEI (Aldrich, 25 kDa) and mPEG (Aldrich, 550 Da): M_n = 69 000, M_w = 1 480 000, M_p = 99 000 and 1100, PD = 2.1, bimodal.

Investigations of the surface activity of the polymer of Example No. 1 were carried out by the method of Lecomte du Nouy (ring method) at 22°C using a tensiometer. The surface tension of the solution in relation to air was measured. The instrument was calibrated with extra pure water, which was also employed as solvent for the polymer sample.
Measured data: σ_{min} = 51 mN/m, CMC = 15 mg/ml.

The following can be prepared in the same way: (all starting compounds are obtainable from Aldrich)

No.	Starting compounds/homopolymers			
	Polyethyleneimine	Hydrophilic, nonionic polymer	Molar ratio PEI: PEG	Structure of the block copolymer
2	lPEI M_n ca. 423	mPEG M_n ca. 550	1:1	AB diblock
3			1:2	ABA triblock
4	bPEI M_w ca. 800	mPEG M_n ca. 550	1:1	AB diblock
5			1:2	ABA triblock
6			1:4	star
7	bPEI M_w ca. 800	mPEG M_n ca. 5000	1:1	AB diblock
8			1:2	ABA triblock
9			1:4	star
10	bPEI M_w ca. 2000	mPEG M_n ca. 550	1:1	AB diblock
11			1:2	ABA triblock
12			1:4	star
13	bPEI M_w ca. 2000	mPEG M_n ca. 5000	1:1	AB diblock
14			1:2	ABA triblock
15	bPEI M_w ca. 25 000	mPEG M_n ca. 550	1:1	AB diblock
16			1:2	ABA triblock
17			1:4	star
18			1:20	star
19	bPEI M_w ca. 25 000	mPEG M_n ca. 2000	1:1	AB diblock
20			1:2	ABA triblock
21			1:4	star
22			1:10	star
23	bPEI M_w ca. 25 000	mPEG M_n ca. 5000	1:1	AB diblock
24			1:2	ABA triblock
25			1:4	star
26			1:10	star

Example 27: Preparation of a $PEG(PEI)_n$ block copolymer

Activation of branched PEG

3.79 g of HMDI (22.54 mmol, 80 eq.) are dissolved in 10 ml of chloroform in a 100 ml round-bottomed flask with magnetic stirring bar, reflux condenser and drying tube on top. A solution of 2 g of an eight-arm branched PEG (bPEG, MW = 10 kDa, 0.2 mmol, 1 eq.) in 20 ml of chloroform is slowly added dropwise to the stirred HMDI solution. The mixture is boiled for 4 hours and then stirred at room temperature for a further 8 hours. The solvent is removed under reduced pressure, and the excess HMDI is extracted with petroleum ether (40-60) (3x50 ml). A reddish oil is obtained in a yield of 58% (1.38 g).

Preparation of a PEG-graft-PEI block copolymer

2.20 g of a branched PEI (bPEI, M_w = 800 Da, M_n = 600 Da, 3.66 mmol, 25 eq.) are dissolved in 20 ml of chloroform in a 100 ml round-bottomed flask with magnetic stirring bar, reflux condenser and drying tube on top. A solution of 1.21 g of the HMDI-activated bPEG (M_n = 8.5 kDa, 0.14 mmol, 1 eq.) in 30 ml of chloroform is slowly added dropwise to the stirred PEI solution at room temperature. The mixture is boiled for 12 hours. The solution is then slowly added dropwise to 500 ml of diethyl ether while stirring. After 12 hours, a viscous yellowish oil has deposited. The cloudy supernatant is discarded, and the oil is dissolved in 50 ml of ethanol. The solution is again added dropwise to 500 ml of diethyl ether, and the oil which has again separated out is isolated by decantation. The product is dissolved in ethanol for filtration, and the solvent is removed in a vacuum oven at 50°C. 1.13 g of a yellowish viscous to resinous oil are obtained (yield: 59%).

The polymers were characterized by ^1H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 27. They are representative of the other examples, for which similar data

were obtained.

^1H NMR (500 MHz, $CDCl_3$): δ/ppm = 1.22 (isocyanate CH_2), 1.36 (isocyanate CH_2), 2.40-2.70 (ethyleneimine CH_2), 3.03 (isocyanate CH_2), 3.19 (isocyanate CH_2), 3.55 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm = 25.9 (isocyanate CH_2), 29.4 (isocyanate CH_2), 39.2 (ethyleneimine CH_2), 41.2 (isocyanate CH_2), 47.0 (ethyleneimine CH_2), 48.9 (ethyleneimine CH_2), 52.0 (ethyleneimine CH_2), 54.2 (ethyleneimine CH_2), 61.1 (isocyanate CH_2), 69.2 and 71.1 and 72.3 (ethylene glycol CH_2), 156.0 (-NHC(O)O-), 162.1 (-NHC(O)NH-).

GPC (aminoethyl methacrylate gel, 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 22 000, M_w = 43 000, M_p = 31 000, PD = 1.9, monomodal. Comparison with blend of 8-arm PEG (Shearwater, 10 kDa) and PEI (Aldrich, 800 Da): M_n = 3100, M_w = 15 000, M_p = 12 000, PD = 4.91, monomodal.

Investigations of the surface activity of the polymer of Example No. 27 were carried out by the method of Lecomte du Nouy (ring method) at 22°C using a tensiometer. The surface tension of the solution in relation to air was measured. The instrument was calibrated with extra pure water, which was also employed as solvent for the polymer sample.
Measured data: σ_{min} = 56 mN/m, CMC = 12 mg/ml.

The following can be prepared in the same way:

No.	Starting compounds/homopolymers		
	Hydrophilic, noninic polymer	Polyethyleneimine	Structure of the block copolymer
28	mPEG M_n ca. 5000 (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB
29		bPEI M_n ca. 800 (Aldrich)	AB
30		bPEI M_n ca. 2000 (Aldrich)	AB
31	lPEG M_n ca. 5000 (Aldrich)	lPEI M_n ca. 423 (Aldrich)	ABA
32		bPEI M_n ca. 800 (Aldrich)	ABA
33		bPEI M_n ca. 2000 (Aldrich)	ABA
34	4-arm PEG MW ca. 15 000 (Shearwater)	lPEI M_n ca. 423 (Aldrich)	AB_4
35		bPEI M_n ca. 800 (Aldrich)	AB_4
36		bPEI M_n ca. 2000 (Aldrich)	AB_4
37	8-arm PEG MW ca. 10 000 (Shearwater)	lPEI M_n ca. 423 (Aldrich)	AB_8
38		bPEI M_n ca. 800 (Aldrich)	AB_8
39		bPEI M_n ca. 2000 (Aldrich)	AB_8
40	Star PEG 429 MW ca. 250 000 (Shearwater)	lPEI M_n ca. 423 (Aldrich)	AB_{13}
41		bPEI M_n ca. 800 (Aldrich)	AB_{13}
42		bPEI M_n ca. 2000 (Aldrich)	AB_{13}
43	α-Cyclodextrin (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB_{18}
44		bPEI M_n ca. 800 (Aldrich)	AB_{18}
45		bPEI M_n ca. 2000 (Aldrich)	AB_{18}
46	β-Cyclodextrin (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB_{21}
47		bPEI M_n ca. 800 (Aldrich)	AB_{21}
48		bPEI M_n ca. 2000 (Aldrich)	AB_{21}
49	γ-Cyclodextrin (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB_{24}
50		bPEI M_n ca. 800 (Aldrich)	AB_{24}
51		bPEI M_n ca. 2000 (Aldrich)	AB_{24}
52	PVA 80% hydrolyzed M_w = 9000-10 000	lPEI M_n ca. 423 (Aldrich)	AB_n
53		bPEI M_n ca. 800 (Aldrich)	AB_n
54		bPEI M_n ca. 2000 (Aldrich)	AB_n

Example 55:

Preparation of a PEG-PEI copolymer (macroregulator route)

1 g (0.2 mmol) of a monomethylated PEG (MW 5000 g/mol) which has an amino group at the other end of the chain is weighed into a 50 ml round-bottomed flask with magnetic stirring bar and reflux condenser, and is dissolved in 20 ml of distilled water. 2 ml (39 mmol) of ethyleneimine are added to this polymer solution. The polymerization is started with 200 µl (2 mmol) of dimethyl sulfate as initiator, and the mixture is heated at 60°C for 8 days. The solvent is then removed under reduced pressure in order to redissolve the remaining mass in 20 ml of ethanol. The solution is added dropwise to 250 ml of diethyl ether, whereupon the polymer separates out. The polymer is isolated by filtration, and solvent residues are removed in the vacuum oven at 50°C for 3 weeks. 1.9 g of a pale yellowish, resinous polymer are obtained (yield: 73%).

The following can be prepared in a similar way: (all amino-modified PEGs are obtainable from RAPP Polymere, Tübingen)

No.	Starting compounds			
	Polyethylene glycol	Polyethylene-imine	Molar ratio EG:EI	Structure of the block copolymer
56	CH_3O-PEG-NH_2 M_n ca. 2000	Ethyleneimine	1:1	AB diblock
57			1:2	AB diblock
58			1:10	AB diblock
59	CH_3O-PEG-NH_2 M_n ca. 5000	Ethyleneimine	1:1	AB diblock
60p			1:10	AB diblock
61	CH_3O-PEG-NH_2 M_n 10 000	Ethyleneimine	1:1	AB diblock
62			1:2	AB diblock
63			1:10	AB diblock
64	CH_3O-PEG-NH_2 M_n 20 000	Ethyleneimine	1:1	AB diblock
65			1:2	AB diblock
66			1:10	AB diblock

The polymers were characterized by ^1H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 56. They are representative of the other examples, for which very similar data were obtained.

^1H NMR (500 MHz, D_2O): δ/ppm = 2.60-3.00 (ethyleneimine CH_2), 3.78 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, D_2O): δ/ppm = 38.2 (ethyleneimine CH_2), 39.9 (ethyleneimine CH_2), 46.2 (ethyleneimine CH_2), 47.9 (ethyleneimine CH_2), 51.7 (ethyleneimine CH_2), 53.4 (ethyleneimine CH_2), 54.8 (ethyleneimine CH_2), 70.2 (ethylene glycol CH_2).

GPC (aminoethyl methacrylate gel, 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 21 000, M_w = 40 000, M_p = 16 000, PD = 1.9, monomodal.

Comparison with CH_3O-PEG-NH_2 (RAPP Polymere, 5000 Da): M_n = 9100, M_w = 14 000, M_p = 16 000, PD = 1.6, monomodal.

Example 67:

Preparation of a PEG-PEI copolymer (macroinitiator route)

Preparation of the macroinitiator

2 g (0.4 mmol, 1 eq.) of a monomethyl ether polyethylene glycol (Aldrich, MW 5000) are weighed into a 50 ml round-bottomed flask with magnetic stirring bar and reflux condenser and are dissolved in 25 ml of distilled chloroform. 0.31 g of tosyl chloride (1.6 mmol, 4 eq.) are added to the stirred polymer solution. Finally, 0.22 ml of triethylamine (0.16 g, 1.6 mmol, 4 eq.) are added to the mixture as catalyst. The mixture is heated under reflux for 18 h. To isolate and purify the polymer, the solution is poured into 500 ml of diethyl ether. The precipitated polymer is filtered off, washed with a large amount of diethyl ether and dried in vacuo. 1.90 g of a white, flaky substance are obtained (91% yield).

Preparation of the PEG-PEI block copolymer

0.5 g of the macroinitiator (0.096 mmol, 1 eq.) is weighed into a 25 ml round-bottomed flask with magnetic stirring bar and reflux condenser and is dissolved in 10 ml of distilled water. While stirring, 1 ml of ethyleneimine (0.832 g, 19.32 mmol, 200 eq.) is added dropwise, and the mixture is heated at 60°C for 24 h. The volatile components are removed under reduced pressure. A white, resinous substance remains and is redissolved in 10 ml of water and precipitated with 200 ml of tetrahydrofuran. The polymer is isolated

by decantation and dried in vacuo. 0.95 g of a yellowish resinous substance is obtained (71% yield).

The following can be prepared in a similar way: (all monomethyl-PEGs are obtainable from Aldrich)

No.	Starting compounds			
	Polyethylene glycol	Polyethyleneimine	Molar ratio PEG:EI	Structure of the block copolymer
68	CH_3O-PEG-Ts M_n ca. 550	Ethyleneimine	1:10	AB diblock
69			1:50	AB diblock
70			1:200	AB diblock
71	CH_3O-PEG-Ts M_n ca. 750	Ethyleneimine	1:10	AB diblock
72			1:50	AB diblock
73			1:200	AB diblock
74	CH_3O-PEG-Ts M_n ca. 2000	Ethyleneimine	1:10	AB diblock
75			1:50	AB diblock
76			1:200	AB diblock
77	CH_3O-PEG-Ts M_n ca. 5000	Ethyleneimine	1:10	AB diblock
78			1:50	AB diblock

The polymers were characterized by ^1H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 67. They are representative of the other examples, for which very similar data were obtained.

^1H NMR (500 MHz, D_2O): δ/ppm = 2.80-3.20 (ethyleneimine CH_2), 3.80 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, D_2O): δ/ppm = 37.9 (ethyleneimine CH_2), 39.4 (ethyleneimine CH_2), 46.1 (ethyleneimine CH_2), 47.2 (ethyleneimine CH_2), 51.3-52.7 (ethyleneimine CH_2), 70.2 (ethylene glycol CH_2).

GPC (aminoethyl methacrylate gel), 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards):
M_n = 35 000, M_w = 90 000, M_p = 52 000, PD = 2.6, monomodal.
Comparison with CH_3O-PEG-Ts 5000 Da): M_n = 4800, M_w = 7600, M_p = 8600, PD = 1.6, monomodal.

Abbreviations

bPEG	branched polyethylene glycol
bPEI	branched polyethyleneimine
CMC	critical micelle concentration
DMF	dimethylformamide
HMDI	hexamethylene diisocyanate
lPEG	linear polyethylene glycol
lPEI	linear polyethyleneimine
M_n	number average molecular weight
M_p	peak molecular weight
mPEG	monomethoxy polyethylene glycol
M_w	weight average molecular weight
MW	unspecified average molecular weight
PD	polydispersity
Ts	tosyl
σ_{min}	minimum surface tension

Biological Examples

I. Transfection experiments

The transfection properties of the polymers $PEI(PEG)_{20}$ (Example 1) and $PEG(PEI)_8$ (Example 27) were studied on the 3T3 cell line. 50 000 cells/well were seeded in 12 well plates and incubated for 24 hours (DMEM + 2 mM glutamine + 10% FCS, 37°C, 10% CO_2). The medium was then changed. 4 µg of pGL3 plasmid in 100 µl of 150 mM saline in each well were complexed with the appropriate amount of polymer in 100 µl of 150 mM saline and, after 10 minutes, added to the cells. After 4 hours, the medium was again changed and, after 48 hours, the evaluation took place. Luciferase expression was determined using the Promega luciferase assay kit in a Berthold Sirius luminometer. The protein concentration was quantified with a modified BCA assay. The stated data are in each case the mean of three wells ± standard deviation for the corresponding nitrogen/phosphorus ratios.

Example 1: [$PEI(PEG)_{20}$]

Measured data:

N/P 5:	0.0057 ± 0.0036 ng/mg of protein
N/P 10:	0.1786 ± 0.1522 ng/mg of protein
N/P 20:	0.6952 ± 0.5498 ng/mg of protein
N/P 50:	5.1963 ± 2.6863 ng/mg of protein
(only plasmid:	0.0000 ± 0.00004 ng/mg of protein)

Example 27: [$PEG(PEI)_8$]

Measured data:

N/P 5:	0.0024 ± 0.0012 ng/mg of protein
N/P 10:	0.0045 ± 0.0046 ng/mg of protein
N/P 20:	0.0109 ± 0.0078 ng/mg of protein
N/P 50:	0.0765 ± 0.0498 ng/mg of protein
(only plasmid:	0.0000 ± 0.00004 ng/mg of protein)

In both cases it was possible to detect gene expression on the basis of transfection having taken place. Moreover, $PEI(PEG)_{20}$ shows a distinctly greater transfection efficiency than does $PEG(PEI)_8$.

II. In vitro cytotoxicity determination by the MTT assay:

The copolymers of Examples 1 and 27 were studied for their cytotoxicity in the cell culture model using the MTT assay by the method of Mosmann (J. Immunol. Methods. 65: 55-63 (1983)). 8000 L929 mouse fibroblasts/well were preincubated in 96 wells for 24 h and treated with the polymer solutions at various concentrations for 3, 12 and 24 h. The mitochondrial activity was determined through the conversion of the MTT dye to the formazan, which was quantified by spectrophotometry. The polymers were employed as solutions in DMEM with 10% FCS in five different concentrations. If necessary, the pH was adjusted to 7.4 and the samples were sterilized by filtration (0.2 µm). The blends were prepared by mixing the two individual components (subtracting the amount of spacer). For the evaluation, the cellular viability [%] was plotted against the polymer concentrations employed, and the IC50 was determined.

Result:

- The in vitro cytotoxicity of the free polymers increases with increasing polymer concentration and with increasing incubation time.
- Copolymer of Example 1: The toxicity of the mixture of individual components PEI 25 kDa and PEG 550 Da corresponds to the toxicity of the free PEI 25 kDa. The tolerability is distinctly improved by the covalent linkage of the two components. Although the toxicity profile after 24 h corresponds to that of the individual components and thus to that of the free PEI 25 kDa, the cytotoxicity falls with shorter incubation periods. The PEG coating masks the positive charge of the polyethyleneimine, and thus the charge-mediated effects on cell membranes are reduced.

- Copolymer of Example 27: The mixture of the two individual components PEI 700 Da and PEG 10 kDa showed no reduction in the viability of the cells up to 10 mg/ml. In the same concentration range, the copolymer showed an increased limitation on cellular viability after 3, 12 and 24 h, which can be explained by the increase in molecular weight.
- Example 27 shows less cytotoxicity than Example 1.

III. In vitro cytotoxicity determination by the LDH assay:

L929 mouse fibroblasts were seeded in the same cell density as in the MTT assay in 6-well multidishes, preincubated for 48 h and incubated with the polymer solution (in PBS pH 7.4) for 1, 2, 3 and 6 h. The extracellular LDH fraction was quantified with a standard kit (Sigma, DG-1340-K) by photometric determination of the reduction of NAD in the presence of lactate and LDH. To determine the 100% value, cells were lyzed with 0.1% Triton X-100.

Result:

The LDH assay confirms the results of the MTT test. Correlation of the two assays shows that membrane damage starts first and, after a time lag, the reduction in metabolic activity starts. The membrane-damaging effect of the polymers becomes stronger as the incubation time and polymer concentration increase.

IV. DNA binding of the copolymers determined by agarose gel electrophoresis

The binding capacity of the copolymers of Examples 1 and 27 was determined by electrophoresis on 1% agarose gels at 80 V. The plasmids (CMV-nlacZ) are located by UV excitation at 254 nm after ethidium bromide staining.

Result:

- Both polymers are capable of electrostatic interaction with the plasmid.
- Consistent with the blend, the polymer of Example 1 is able to bind plasmid completely from a nitrogen-PEI/phosphate-DNA ratio (N/P ratio) of 1.7 onwards. The ethidium bromide exclusion observed with the blend (from N/P 5.8), a sign of intensive DNA condensation, is incomplete for the copolymer up to N/P 23.0.
- Whereas for the blend of Example 27 complete plasmid binding is to be observed only from N/P 4.1 onwards, and no complete ethidium exclusion is to be observed, the copolymer showed plasmid binding from N/P 2.4 onwards and exclusion of the dye from N/P 16.6 onwards.

V. Erythrocyte aggregation assay

Erythrocytes were isolated from the citrated blood of Wistar rats by the method of Parnham and Wetzig (Chem. Phys. Lipids, 1993, 64: 263-274), seeded in 24 wells and incubated with the test solutions at 37°C for 2 h. The aggregation and adhesion of the erythrocytes under the influence of the polymer were examined under the microscope. Untreated erythrocytes served as control.

Result:

- Free copolymer of Example 1 showed at concentrations of 0.27-18 µg/well by comparison with the blend and with PEI 25 kDa a reduced aggregation and adhesion of the red blood corpuscles to the cell culture dishes. Whereas no significant differences were to be seen at low concentrations (0.27-0.7 µg/well), a marked difference between copolymer and blend or PEI 25 kDa was detectable with increasing concentration. The aggregating effect increases as the N/P ratio increases.
- Copolymer of Example 27 showed the opposite behavior. Aggregation of the blend and of free PEI is less pronounced than that of the

copolymer.

- The erythrocyte aggregation is significantly reduced through complexation of both copolymers with plasmid DNA compared with the free polymer.

VI. Hemolysis assay

Erythrocytes were isolated from the citrated blood of Wistar rats by the method of Parnham and Wetzig (Chem. Phys. Lipids, 1993, 64: 263-274), mixed with the polymer solutions and incubated at 37°C for 1 h. The erythrocytes are pelleted by centrifugation (10 min, 25°C, 700 g), and the hemolyzate is measured by photometry on the supernatant at 540 nm.

Result:

- The individual components PEG 8-arm, PEG 500 Da and PEI 700 Da show no significant hemolytic effects in the concentration range 0.001-10 mg/ml (all 1-3%).
- The copolymer of Example 27 likewise shows no pronounced effects (<5%) in the same concentration range.
- With the individual components PEI 25 kDa and with the blend for Example 1, the hemolytic activity increases at 0.001-10 mg/ml (22.13% at 10 mg/ml).
- The copolymer of Example 1 shows an increasing lytic activity of up to 13.30% up to 0.5 mg/ml, while the hemolytic effect decreases again at higher concentrations up to 10 mg/ml (2.90% at 10 mg/ml).

VII. Pharmacokinetics and organ distribution of polymer-DNA complexes in mice

The pharmacokinetics and organ distribution of the copolymers of Example 1 and 27 were determined in balb/c mice. The polymers were radiolabeled with ^{125}I Bolton Hunter reagent (Pharmacia Biotech). Amounts of 0.4 or 0.04

or 0.008 mg of PEI (component) per kg of mouse were complexed with the appropriate amount of NF-κB decoy oligodeoxynucleotide (ODN) in the nitrogen/phosphorus ratio N/P 3.5 or N/P 6 in a total volume of 80 µl in 5% glucose solution and, after 10 minutes, injected into the anesthetized mice via the subclavian vein. After 20 seconds, 1, 2, 5, 15, 30, 60, 90 and 120 minutes, blood samples were taken from the arteria aorta communis through a catheter. The urine was collected through a bladder catheter for 120 minutes. After 120 minutes, the mice were decapitated and the organs cortex, kidney, liver, heart, lung, spleen and adipose tissue were removed. The amount of polymer in the samples was determined by measuring the radioactivity with a 1277 Gammamaster automatic gamma counter (LKB Wallac).

The data were analyzed using the Kinetica 1.1 program and a 2-compartment model for i.v. bolus injection. The volume of distribution (V_c), the elimination constant (k_{el}) and AUC were calculated from the blood level plots. Mean ± standard deviation are stated when three animals could be analyzed, the median is stated for two animals, and the value is stated in parentheses when there was only one animal.

Complex preparation and dosages

Polymer	N/P	Dose [mg/ kg]	V_c [ml]	k_{el} [min^{-1}]	AUC [min µg ml^{-1}]
25 kDa PEI	3.5:1	0.4	23.39	0.106	4.89
Example 1	3.5:1	0.4	(4.54)	(0.028)	(79.03)
Example 27	3.5:1	0.4	5.84±0.4	0.104±0.017	16.86±1.64

25 kDa PEI	6:1	0.4	5.39	0.099	19.22
25 kDa PEI	6:1	0.04	1.37±0.2	0.14±0.026	6.22±1.18
25 kDa PEI	6:1	0.008	9.57±1.78	0.063±0.009	0.34±0.1
Example 1	6:1	0.4	6.20	0.067	27.84
Example 1	6:1	0.04	3.37±0.32	0.072±0.01	4.0±0.67
Example 1	6:1	0.008	5.1±0.55	0.054±0.004	0.80±0.10
Example 27	6:1	0.4	8.12	0.0593	21.72

Result:

- Observations with a relatively low dose indicate that the toxicity of $PEI(PEG)_{20}$ is weaker than that of PEI 25 kDa.
- The plasma levels of all the polymers could be described by a 2-compartment model.
- The copolymers have a higher AUC and a smaller volume of distribution than the 25 kDA PEI. $PEI(PEG)_{20}$ (Example 1) has a larger effect than $PEG(PEI)_8$ (Example 27).
- Elimination was reduced with the copolymers.
- V_c and k_{el} show no detectable dose-dependency.
- The calculated AUC for PEI 25 kDa and Example 1 was proportional to the dose, while the gradient of the AUC/dose lines was larger with the copolymer of Example 1.
- The main organs of distribution after 120 minutes were liver, kidney and spleen. For the 6:1 complexes, the copolymers show a reduced uptake in liver and spleen and a higher uptake in the kidney compared with PEI 25 kDa.

Claims:

1. A compound of the formula I or II

(I) $A(-X-B)_n$ (II) $C(-Y-D)_m$

in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 100 to 10 000 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 100 to 1 000 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 1 to 20,

-OC(O)NH(aryl)NHC(O)- with aryl = aromatic unit,

$-O(CH_2)_pC(O)-$ with p = 1 to 10,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 20;

n is an integer from 1 to 200;

C is a linear or branched PEI with a molecular weight of from 100 to 1 000 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 3 to 25 000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit,

$-(CH_2)_tC(O)O-$ with t = 2 to 10,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 20,

and

m is an integer from 1 to 200,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby.

2. A compound as claimed in claim 1, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 1000 to 100 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 100 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 2 to 10,

-OC(O)NH(aryl)NHC(O)- with aryl = aromatic unit with one nucleus,

$-O(CH_2)_pC(O)-$ with p = 1 to 3,

$-OCH_2CH(OH)CH_2-$,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 6,

n is an integer from 1 to 50,

C is a linear or branched PEI with a molecular weight of from 400 to 100 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 10 to 5000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit with one nucleus,

$-(CH_2)_tC(O)O-$ with t = 2 to 3,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 6;

and

m is an integer from 1 to 100,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby.

3. A compound as claimed in claim 1, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 5000 to 50 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 50 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 4 to 6,

-OC(O)NH(aryl)NHC(O)- with aryl = tolyl,

$-O(CH_2)_pC(O)-$ with p = 1,

$-OCH_2CH(OH)CH_2-$,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 3;

n is an integer from 1 to 12;

C is a linear or branched PEI with a molecular weight of from 400 to 50 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 10 to 1000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = tolyl,

$-(CH_2)_tC(O)O-$ with t = 2,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 3;

and

m is an integer from 1 to 50,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby.

4. A compound as claimed in claim 1, which has formula I.

5. A compound as claimed in claim 1, which has formula II.

6. A compound as claimed in claim 1, in which X is a linker of the formula $-OC(O)NH(CH_2)_oNHC(O)-$.

7. The method of complexation of polynucleic acids in aqueous systems which comprises contacting a compound of the formula II in which Y is a linker of the formula $-C(O)NH(CH_2)_sNHC(O)O-$ with s = 1-10, and the other radicals are as defined in claim 1 with a polynucleic acid.

8. A process for preparing a compound of the formula I as claimed in claim 1, which comprises

a) reacting compounds of the general formula V

(V) $A-(OH)_n$ with A and n = as in formula I

with diisocyanate, or

b) adding compounds of the general formula VI

(VI) $A\text{-}(NH_2)_n$ (with A and n = as defined in formula I)

to the reaction mixture for the polymerization of ethyleneimine before the start of the polymerization or not until the polymerization is in progress, or

c) employing compounds of the general formula VII

(VII) $A\text{-}(OS(O)_2R^4)_n$ with A as in formula I and R^4 = aliphatic or aromatic radical as macroinitiator for the polymerization of ethyleneimine.

9. A process for preparing compounds of the formula II as claimed in claim 1, which comprises initially reacting compounds of the general formula IX

(IX) D-OH (with D as defined in formula II)

with diisocyanate and subsequently reacting the resulting compound with linear or branched polyethyleneimine.

(VIII)

10. The method of complexation of polynucleic acids in aqueous systems which comprises contacting a compound of the formula I or II

(I) $A(\text{-}X\text{-}B)_n$ (II) $C(\text{-}Y\text{-}D)_m$

in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 100 to 10 000 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 100 to 1 000 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 1 to 20,

-OC(O)NH(aryl)NHC(O)- with aryl = aromatic unit,

$-O(CH_2)_pC(O)-$ with p = 1 to 10,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 20;

n is an integer from 1 to 200;

C is a linear or branched PEI with a molecular weight of from 100 to 1 000 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 3 to 25 000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal is linked to a nitrogen

atom of the PEI:

$-C(O)NH(CH_2)_sNHC(O)O-$ with s = 1 to 20,

$-C(O)NH(aryl)NHC(O)O-$ with aryl = aromatic unit,

$-(CH_2)_tC(O)O-$ with t = 2 to 10,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 20,

and

m is an integer from 1 to 200,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby with a polynucleic acid.

11. The method as claimed in claim 10, wherein a compound of the formula I or II, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 1000 to 100 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 100 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 2 to 10,

-OC(O)NH(aryl)NHC(O)- with aryl = aromatic unit with one nucleus,

$-O(CH_2)_pC(O)-$ with p = 1 to 3,

$-OCH_2CH(OH)CH_2-$,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 6,

n is an integer from 1 to 50,

C is a linear or branched PEI with a molecular weight of from 400 to 100 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 10 to 5000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-C(O)NH(CH_2)_sNHC(O)O-$ with s = 2 to 10,

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit with one nucleus,

$-(CH_2)_tC(O)O-$ with t = 2 to 3,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 6;

and

m is an integer from 1 to 100,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby,

Is used.

12. The method as claimed in claim 10, wherein a compound of the formula I or II, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 5000 to 50 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 50 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 4 to 6,

-OC(O)NH(aryl)NHC(O)- with aryl = tolyl,

$-O(CH_2)_pC(O)-$ with p = 1,

$-OCH_2CH(OH)CH_2-$,

$-OC(O)-$, or

$-O(CH_2)_q$ with q = 1 to 3;

n is an integer from 1 to 12;

C is a linear or branched PEI with a molecular weight of from 400 to 50 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 10 to 1000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-C(O)NH(CH_2)_sNHC(O)O-$ with s = 4 to 6,

-C(O)NH(aryl)NHC(O)O- with aryl = tolyl,

$-(CH_2)_tC(O)O-$ with t = 2,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 3;

and

m is an integer from 1 to 50,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby,

is used.

13. The method of claim 10, wherein the polynucleic acid is DNA.

14. The method of claim 10, wherein the polynucleic acid is RNA.

15. The method of claim 12, wherein the polynucleic acid is a ribozyme.

16. A compostion which comprises at least one nucleic acid and one compound of the formula I or II which is as defined in claim 7.

Abstract

The invention relates to cationic block copolymers of formula A(-X-B)$_n$ or C(-Y-D)$_m$, wherein A represents a hydrophilic polymer, B represents polyethyleneimine (PEI), X represents a bridge, n represents 1-200, C represents PEI, D represents the residue of a polyethylene glycol, Y represents a bridge and m represents 1-200. The invention also relates to methods for producing the inventive cationic block polymers and to their use e.g. as a tenside and for complexing nucleic acids.

Cationic block copolymers

Description

WO 98/59064 discloses PEI-PEG block copolymers and their use as vehicles for transporting nucleic acid into higher eukaryotic cells. The described copolymer was composed of branched PEI and linear PEG. PEI was PEGylated using methoxy-succinimidyl-propionate-PEG.

S. V. Vinogradov, T. K. Bronich and A. V. Kabanov (Bioconjugate Chem. 1998, 9, 805-812) describe the preparation of PEI-PEG and polyspermine-PEG block copolymers by using branched PEI and branched polyspermines through a coupling reaction with a monomethoxy-PEG activated with 1,1'-carbonyl-diimidazole. The copolymers were used for complexation with oligonucleotides.

L. M. Bronstein, M. Antonietti et al. (Inorganica Chimica Acta 1998, 280, 348-354) describe PEI-PEG block copolymers and their preparation by coupling of branched PEI with monomethoxy-PEG which has a terminal acid chloride function, and the use thereof for preparing metal colloids.

V. Toncheva et al. (Biochimica et Biophysika Acta 1998, 138, 354-358) relates to block copolymers consisting of poly(L-lysine) and a plurality of hydrophilic polymers, such as PEG, dextran and poly(N-(2-hydroxypropyl)-methacrylamide, processes for their preparation and their use as vehicles for nucleic acid gene transfer.

2. In all cases, for this purpose the reactive terminus of the hydrophilic, nonionic polymer was activated for the coupling reaction with the cationic polymer by a reagent which represents a linker between the blocks in the copolymer which is generated.

3. The hydrophilic, nonionic polymer was in all cases a linear polymer.

In addition, US-A 5 204 196 discloses polymers which contain a PEI backbone to which a polyethylene glycol ether of the formula $-O(CH_2-CH_2)_n-CH_3$ is linked by means of an isocyanate. These polymers are used in combination with inorganic salts as solid electrical conductor.

Novel cationic block copolymers of the general formula I and II

(I) $A(-X-B)_n$

(II) $C(-Y-D)_m$

have been found, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 100 to 10 000 000 g/mol, preferably from 1000 to 100 000 g/mol and in particular from 5000 to 50 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 100 to 1 000 000 g/mol, preferably from 400 to 100 000 g/mol and in particular from 400 to 50 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 1 to 20, preferably 2 to 10, in particular 4 to 6,

$-OC(O)NH(aryl)NHC(O)-$ with aryl = aromatic unit with, preferably, 6-14 C atoms consisting of one or more aromatic nuclei which are connected together in fused or in polyphenylic form, preferably with one nucleus, in particular tolyl,

$-O(CH_2)_pC(O)-$ with p = 1 to 10, preferably 1 to 3, in particular 1,

$-OCH_2CH(OH)CH_2-$,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 20, preferably 1 to 6, in particular 1 to 3;

n is an integer from
1) 1 to 200, preferably
2) 1 to 50,
3) 1 to 12,
4) 1 to 8 or, particularly preferably,
5) 2 to 8;

C is a linear or branched PEI with a molecular weight of from 100 to 1 000 000 g/mol, preferably from 400 to 100 000 g/mol, and in particular from 400 to 50 000 g/mol;

D is a residue of a polyethylene glycol which is linked via O of the formula

$-(CH_2CH_2O)_{n'}-R^1$

in which n′ is from 3 to 25 000, preferably from 10 to 5000 and in particular from 10 to 1000, and R^1 is hydrogen, an aliphatic radical such as (C_1-C_6)-alkyl (methyl, ethyl, tert-butyl and the like) or another OH-protective group such as acyl (e.g. optionally substituted benzoxycarbonyl), optionally substituted benzyl, picolyl, or a cellular ligand in order to bring about specific uptake of a nucleic acid-copolymer complex through binding to cell surface proteins, in particular

receptors;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit with, preferably, 6 to 14 C atoms consisting of one or more aromatic nuclei which are connected together in fused or in polyphenylic form, preferably with one nucleus, in particular tolyl,

$-(CH_2)_tC(O)O-$ with t = 2 to 10, preferably 2 to 3, in particular 2,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 20, preferably 1 to 6, in particular 1 to 3;

and

m is an integer from
1) 1 to 200, preferably
2) 1 to 100, in particular
3) 1 to 50,

with the proviso that the radicals and variables in formula II are defined in such a way that no compounds of the formula I are included thereby.

The cationic block copolymers of the invention differ from the known block copolymers in at least one of the

following three features:

1. A hydrophilic, nonionic polymer is equipped with side arms of a cationic polymer.
2. The linkers differ from those of known block copolymers.
3. They have a branched hydrophilic, nonionic polymer.

A preferably means linear or branched polymers which are composed of carbon and oxygen and which may, where appropriate, also comprise cyclic, star or dendritic structures, such as, for example, residues of linear PEG, multi-arm branched PEG, star PEG, polysaccharides including cyclodextrins, PVA, arborols (dendrimers with terminal hydroxyl groups),

w and w' are identical or different integers which are chosen so as to balance the positive charges in the radicals of the formulae III and IV.

The polyethyleneimines can be prepared in a manner known per se or are commercially available under the BASF brand name Lupasol® or under the name polyethyleneimine or ethyleneimine polymer in various molecular weights of from 400 to 2 000 000 g/mol (from Aldrich, Sigma, Fluka or directly from BASF). Preference is given to polyethyleneimines with a molecular weight of from 400 to 2000 g/mol for B and to polyethyleneimines with a molecular weight of from 400 to 800 000 g/mol, particularly preferably from 400 to 25 000 g/mol, for C.

The groups described under D are residues of polyethylene glycols which are protected on one terminus by a radical R^1 such as, for example, methyl or another suitable protective group. However, R^1 may also be a group which performs a specific or nonspecific biological function, in particular a ligand for interactions with receptors for target cell-specific uptake of a block copolymer-active substance complex into higher eukaryotic cells and the cell nucleus thereof, where the active substance is preferably an oligonucleotide or a gene (gene targeting). R^1 can thus also be a ligand for a specific interaction and uptake into target organ tissue or cells, for example proteins, in particular antibodies or antibody fragments such as Fab, $F(ab)_2$, scFv, cytokines or lymphokines, such as interleukins (IL-2 to x), interferon GM-CSF,

growth factors such as EGF, PDGF, FGF, EPO,
integrins such as ICAM, VCAM or
glycoproteins such as lectins or glycosilated proteins (see above) or
lipoproteins such as LDL, HDL or
transporter proteins such as transferrin or
peptides such as LH-RH, calcitonin, oxytocin, insulin, somatostatin, IGF, RGD or
carbohydrates such as galactose, mannose, glucose, lactose or
hormones such as steroids, THR or
vitamins such as B_{12}, folic acid.

The invention also relates to processes for preparing compounds of the formula I, which comprise

a) reacting compounds of the general formula V

(V) $A-(OH)_n$ with A and n = as in formula I

with diisocyanate, preferably hexamethylene diisocyanate, and reacting the compound resulting therefrom with polyethyleneimines with the general formulae III and IV, or

b) adding compounds of the general formula VI

(VI) $A-(NH_2)_n$ (with A and n = as defined in formula I)

to the reaction mixture for the polymerization of ethyleneimine before the start of the polymerization or not until the polymerization is

in progress, or

c) employing compounds of the general formula VII

(VII) $A-(OS(O)_2R^4)_n$ with A as in formula I and R^4 = aliphatic or aromatic radical, preferably p-tolyl, fluoride, trifluoromethyl or methyl,

as macroinitiator for the polymerization of ethyleneimine.

Compounds of the formula VI are commercially available in various molecular weights, for example from Shearwater.

Compounds of the general formula VII are obtained by reacting compounds of the general formula V with compounds of the general formula VIII

(VIII) $Cl-S(O)_2R^4$ (R^4 as defined above).

The invention further relates to processes for preparing compounds of the formula II, which comprise

d) initially reacting compounds of the general formula IX

(IX) D-OH (with D as defined in formula II)

with diisocyanate, preferably hexamethylene diisocyanate, and subsequently reacting the resulting compound with linear or branched polyethyleneimine. Protective groups introduced

where appropriate to protect OH groups can be eliminated in a manner known per se (see, for example, Büllesbach, Kontakte (Merck) 1/1980, pp. 23 et seq.).

The process described under a) is preferably carried out in such a way that a 4- to 20-fold excess of diisocyanate, preferably hexamethylene diisocyanate, is employed per terminal hydroxyl group of the polymer block A. The reaction is carried out in chloroform at temperatures from room temperature to the boiling point of the solvent, but preferably at the boiling point of the solvent. The chosen reaction time is between 2 and 24 hours, but preferably 4 hours. The polymer concentration in the reaction mixture is between 10 g/l and 500 g/l, preferably 100 g/l. The product is isolated by removing the solvent under reduced pressure and removing the excess diisocyanate by repeated extraction with petroleum ether (boiling range: 40-60°C). This intermediate is reacted with a 3- to 10-fold excess of PEI macromolecules per terminal hydroxyl group of the starting compound. The reaction is carried out in chloroform at temperatures from room temperature to the boiling point of the solvent, but preferably at the boiling point of the solvent. The chosen reaction time is between 6 and 72 hours, but preferably 12 hours. The polymer concentrations, both those of the PEI and those of the nonionic hydrophilic polymer which has been activated with hexamethylene diisocyanate, in the reaction mixture are between 10 g/l and 500 g/l, preferably between 30-200 g/l. The product is isolated by precipitating the polymer in a 10-30-fold volumetric excess of diethyl ether. Excess PEI can be removed from

the block copolymer by repeated reprecipitation with ethanol and diethyl ether as solvent.

The process described under b) is carried out by mixing the ethyleneimine and the amino-terminated hydrophilic nonionic polymer in water in a concentration of from 10 g/l to 500 g/l in each case. The molar ratio of the two components is between 1:10 to 1:10 000. The ethyleneimine polymerization is then initiated by adding a suitable catalyst, for example hydrochloric acid, and the mixture is brought to a temperature of 40-100°C. The copolymer is generated by a chain termination reaction. The block copolymer is repeatedly reprecipitated with the aid of suitable solvents, for example ethanol and diethyl ether, and/or by pressure filtration, to remove PEI homopolymer which is a possible by-product. One variation of this preparation process comprises adding the amino-terminated hydrophilic nonionic polymer to the hot polymerization mixture only after a certain reaction time of from 30 minutes up to 72 hours.

The process described under c) is carried out by reacting the terminal hydroxyl group(s) of the polymer block A with a sulfonyl chloride of the general formula VIII, but especially with toluenesulfonyl chloride (tosyl chloride). This reaction is carried out in aqueous and/or polar organic solvent, preferably in a water/tetrahydrofuran mixture, at temperatures of from -10°C to the boiling point of the solvent, preferably at temperatures of from -10°C to the boiling point of the solvent, preferably at temperatures of from 0°C to 25°C, and (if necessary) in the presence of catalysts

such as, for example, triethylamine or sodium hydroxide. The product is isolated by removing the solvent under reduced pressure. This polymer is subsequently used as macroinitiator for the ethyleneimine polymerization. For this purpose, the product with the general formula VII is reacted with ethyleneimine in aqueous or polar organic solvent at temperatures of from 0°C to the boiling point of the solvent. The molar ratio of the two components is between 1:10 to 1:10 000. No by-product is formed in this reaction. The final product can be isolated by precipitating the polymer in a suitable solvent such as, for example, diethyl ether. The process described under d) is preferably carried out by reacting a compound of the general formula IX with a small excess, preferably a 2- to 10-fold excess, of diisocyanate, preferably hexamethylene diisocyanate. The reaction is carried out in chloroform at temperatures of from 20°C to the boiling point of the solvent, but preferably at the boiling point of the solvent. The chosen reaction time is between 2 and 24 hours, but preferably 10 to 14 hours. The polymer concentration in the reaction mixture is between 10 g/l and 500 g/l, preferably 30 to 150 g/l. The product is isolated by removing the solvent under reduced pressure and removing the excess diisocyanate by repeated extraction with petroleum ether (boiling range: 40-60°C). This intermediate is reacted with PEI macromolecules in a molar ratio of from 1:1 to 100:1. The reaction is carried out in chloroform and, if necessary, with addition of dimethylformamide at temperatures from room temperature to the boiling point of the solvent, but preferably at 60-70°C. The chosen reaction time is between 6 and

In addition, the block copolymers can also be used

- in adhesive and coating systems as additive
- as fixing agents to improve paper strength
- as primers for polymer composite systems such as, for example, multilayer packaging sheets
- for modifying plastics (improving the dyeability, paintability, barrier effect)
- for fixing reactive dyes on cotton
- as coagulant and dispersant for fine suspended particles in industrial waste waters
- for binding heavy metal salts

- for dispersing organic and inorganic pigments
- as addition in ceramic and cement components
- for a wide variety of functions in skin and hair cosmetics and in the dental sector
- for immobilizing medicinal active substances or bioactive compounds on surfaces
- for filtering endotoxins and pathogens out of blood plasma
- for penetration through mucous membranes.

In addition, in aqueous systems the block copolymers form complexes with polynucleic acids such as DNA and RNA, including ribozymes. This property makes them suitable as vehicles or vectors for gene transfer (penetration through cell membranes and translocation into the cell nucleus). They can therefore be used in transfection experiments, in gene therapy and diagnosis (see "Biological Examples" section).

The invention therefore also relates in particular to the use of compounds of the formula I or II which are as defined above and in which additionally Y can be $-C(O)NH(CH2)_sNHC(O)O-$ with s = 1 to 20, preferably 2 to 10, in particular 4 to 6, for the complexation of polynucleic acids such as DNA, RNA and ribozymes, in aqueous systems, and to compositions which comprise at least one such nucleic acid and one compound of the formula I or II.

The invention also relates to the use of the compounds of the formula I or II defined in the preceding paragraph as surfactants.

The following examples serve to illustrate the invention without intending to restrict it thereto.

60-70°C for 12 hours. The mixture is then added dropwise to 500 ml of diethyl ether. After two hours, a viscous yellowish oil has deposited. The cloudy supernatant is discarded, and the oil is dissolved in 30 ml of ethanol. The solution is again added dropwise to 500 ml of diethyl ether, and the oil which has again separated out is isolated by decantation. The product is dissolved in ethanol for filtration, and the solvent is removed in a vacuum oven at 50°C. 2.8 g of a yellowish viscous to resinous oil are obtained (yield: 45%).

The polymers were characterized by ^{1}H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 1. They are representative of the other examples, for which similar data were obtained.

^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm = 1.17 (isocyanate CH_2), 1.26 (isocyanate CH_2), 2.30-2.72 (ethyleneimine CH_2), 2.96 (isocyanate CH_2), 3.15 (isocyanate CH_2), 3.49 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm 14.3 (isocyanate CH_2), 26.2 (isocyanate CH_2), 29.6 (isocyanate CH_2), 36.2 (isocyanate CH_2), 37.5 (ethyleneimine CH_2), 39.1 (ethyleneimine CH_2), 41.1 (isocyanate CH_2), 47.2 (ethyleneimine CH_2), 48.9 (ethyleneimine CH_2), 52.8 (ethyleneimine CH_2), 54.1 (ethyleneimine CH_2), 58.7 (isocyanate CH_2), 69.3 and 70.2 and 71.6 (ethylene glycol CH_2), 156.2 (-NHC(O)O-), 161.7 (-NHC(O)NH-).

GPC (aminoethyl methacrylate gel, 1% formic acid,

0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 8800, M_w = 1 640 000, M_p = 85 000, PD = 19.6, monomodal.
Comparison with blend of PEI (Aldrich, 25 kDa) and mPEG (Aldrich, 550 Da): M_n = 69 000, M_w = 1 480 000, M_p = 99 000 and 1100, PD = 2.1, bimodal.

Investigations of the surface activity of the polymer of Example No. 1 were carried out by the method of Lecomte du Nouy (ring method) at 22°C using a tensiometer. The surface tension of the solution in relation to air was measured. The instrument was calibrated with extra pure water, which was also employed as solvent for the polymer sample.
Measured data: σ_{min} = 51 mN/m, CMC = 15 mg/ml.

The following can be prepared in the same way: (all starting compounds are obtainable from Aldrich)

No.	Starting compounds/homopolymers			
	Polyethyleneimine	Hydrophilic, nonionic polymer	Molar ratio PEI: PEG	Structure of the block copolymer
2	IPEI M_n ca. 423	mPEG M_n ca. 550	1:1	AB diblock
3			1:2	ABA triblock
4	bPEI M_w ca. 800	mPEG M_n ca. 550	1:1	AB diblock
5			1:2	ABA triblock
6			1:4	star
7	bPEI M_w ca. 800	mPEG M_n ca. 5000	1:1	AB diblock
8			1:2	ABA triblock
9			1:4	star
10	bPEI M_w ca. 2000	mPEG M_n ca. 550	1:1	AB diblock
11			1:2	ABA triblock
12			1:4	star
13	bPEI M_w ca. 2000	mPEG M_n ca. 5000	1:1	AB diblock
14			1:2	ABA triblock
15	bPEI M_w ca. 25 000	mPEG M_n ca. 550	1:1	AB diblock
16			1:2	ABA triblock
17			1:4	star
18			1:20	star
19	bPEI M_w ca. 25 000	mPEG M_n ca. 2000	1:1	AB diblock
20			1:2	ABA triblock
21			1:4	star
22			1:10	star
23	bPEI M_w ca. 25 000	mPEG M_n ca. 5000	1:1	AB diblock
24			1:2	ABA triblock
25			1:4	star
26			1:10	star

Example 27: Preparation of a $PEG(PEI)_n$ block copolymer

Activation of branched PEG

3.79 g of HMDI (22.54 mmol, 80 eq.) are dissolved in 10 ml of chloroform in a 100 ml round-bottomed flask with magnetic stirring bar, reflux condenser and drying tube on top. A solution of 2 g of an eight-arm branched PEG (bPEG, MW = 10 kDa, 0.2 mmol, 1 eq.) in 20 ml of chloroform is slowly added dropwise to the stirred HMDI solution. The mixture is boiled for 4 hours and then stirred at room temperature for a further 8 hours. The solvent is removed under reduced pressure, and the excess HMDI is extracted with petroleum ether (40-60) (3x50 ml). A reddish oil is obtained in a yield of 58% (1.38 g).

Preparation of a PEG-graft-PEI block copolymer

2.20 g of a branched PEI (bPEI, M_w = 800 Da, M_n = 600 Da, 3.66 mmol, 25 eq.) are dissolved in 20 ml of chloroform in a 100 ml round-bottomed flask with magnetic stirring bar, reflux condenser and drying tube on top. A solution of 1.21 g of the HMDI-activated bPEG (M_n = 8.5 kDa, 0.14 mmol, 1 eq.) in 30 ml of chloroform is slowly added dropwise to the stirred PEI solution at room temperature. The mixture is boiled for 12 hours. The solution is then slowly added dropwise to 500 ml of diethyl ether while stirring. After 12 hours, a viscous yellowish oil has deposited. The cloudy supernatant is discarded, and the oil is dissolved in 50 ml of ethanol. The solution is again added dropwise to 500 ml of diethyl ether, and the oil which has again separated

out is isolated by decantation. The product is dissolved in ethanol for filtration, and the solvent is removed in a vacuum oven at 50°C. 1.13 g of a yellowish viscous to resinous oil are obtained (yield: 59%).

The polymers were characterized by ^{1}H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 27. They are representative of the other examples, for which similar data were obtained.

^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm = 1.22 (isocyanate CH_2), 1.36 (isocyanate CH_2), 2.40-2.70 (ethyleneimine CH_2), 3.03 (isocyanate CH_2), 3.19 (isocyanate CH_2), 3.55 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm = 25.9 (isocyanate CH_2), 29.4 (isocyanate CH_2), 39.2 (ethyleneimine CH_2), 41.2 (isocyanate CH_2), 47.0 (ethyleneimine CH_2), 48.9 (ethyleneimine CH_2), 52.0 (ethyleneimine CH_2), 54.2 (ethyleneimine CH_2), 61.1 (isocyanate CH_2), 69.2 and 71.1 and 72.3 (ethylene glycol CH_2), 156.0 (-NHC(O)O-), 162.1 (-NHC(O)NH-).

GPC (aminoethyl methacrylate gel, 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 22 000, M_w = 43 000, M_p = 31 000, PD = 1.9, monomodal. Comparison with blend of 8-arm PEG (Shearwater, 10 kDa) and PEI (Aldrich, 800 Da): M_n = 3100, M_w = 15 000, M_p = 12 000, PD = 4.91, monomodal.

Investigations of the surface activity of the polymer of Example No. 27 were carried out by the method of

Lecomte du Nouy (ring method) at 22°C using a tensiometer. The surface tension of the solution in relation to air was measured. The instrument was calibrated with extra pure water, which was also employed as solvent for the polymer sample.

Measured data: σ_{min} = 56 mN/m, CMC = 12 mg/ml.

The following can be prepared in the same way:

No.	Starting compounds/homopolymers		
	Hydrophilic, noninic polymer	Polyethyleneimine	Structure of the block copolymer
28	mPEG M_n ca. 5000 (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB
29		bPEI M_n ca. 800 (Aldrich)	AB
30		bPEI M_n ca. 2000 (Aldrich)	AB
31	lPEG M_n ca. 5000 (Aldrich)	lPEI M_n ca. 423 (Aldrich)	ABA
32		bPEI M_n ca. 800 (Aldrich)	ABA
33		bPEI M_n ca. 2000 (Aldrich)	ABA
34	4-arm PEG MW ca. 15 000 (Shearwater)	lPEI M_n ca. 423 (Aldrich)	AB_4
35		bPEI M_n ca. 800 (Aldrich)	AB_4
36		bPEI M_n ca. 2000 (Aldrich)	AB_4
37	8-arm PEG MW ca. 10 000 (Shearwater)	lPEI M_n ca. 423 (Aldrich)	AB_8

38		bPEI M_n ca. 800 (Aldrich)	AB_8
39		bPEI M_n ca. 2000 (Aldrich)	AB_8
40	Star PEG 429 MW ca. 250 000 (Shearwater)	lPEI M_n ca. 423 (Aldrich)	AB_{13}
41		bPEI M_n ca. 800 (Aldrich)	AB_{13}
42		bPEI M_n ca. 2000 (Aldrich)	AB_{13}
43	α-Cyclodextrin (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB_{18}
44		bPEI M_n ca. 800 (Aldrich)	AB_{18}
45		bPEI M_n ca. 2000 (Aldrich)	AB_{18}
46	β-Cyclodextrin (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB_{21}
47		bPEI M_n ca. 800 (Aldrich)	AB_{21}
48		bPEI M_n ca. 2000 (Aldrich)	AB_{21}
49	γ-Cyclodextrin (Aldrich)	lPEI M_n ca. 423 (Aldrich)	AB_{24}
50		bPEI M_n ca. 800 (Aldrich)	AB_{24}
51		bPEI M_n ca. 2000 (Aldrich)	AB_{24}
52	PVA 80% hydrolyzed M_w = 9000-10 000	lPEI M_n ca. 423 (Aldrich)	AB_n
53		bPEI M_n ca. 800 (Aldrich)	AB_n
54		bPEI M_n ca. 2000 (Aldrich)	AB_n

Example 55:

Preparation of a PEG-PEI copolymer (macroregulator route)

1 g (0.2 mmol) of a monomethylated PEG (MW 5000 g/mol) which has an amino group at the other end of the chain is weighed into a 50 ml round-bottomed flask with magnetic stirring bar and reflux condenser, and is dissolved in 20 ml of distilled water. 2 ml (39 mmol) of ethyleneimine are added to this polymer solution. The polymerization is started with 200 μl (2 mmol) of dimethyl sulfate as initiator, and the mixture is heated at 60°C for 8 days. The solvent is then removed under reduced pressure in order to redissolve the remaining mass in 20 ml of ethanol. The solution is added dropwise to 250 ml of diethyl ether, whereupon the polymer separates out. The polymer is isolated by filtration, and solvent residues are removed in the vacuum oven at 50°C for 3 weeks. 1.9 g of a pale yellowish, resinous polymer are obtained (yield: 73%).

The following can be prepared in a similar way: (all amino-modified PEGs are obtainable from RAPP Polymere, Tübingen)

No.	Starting compounds			
	Polyethylene glycol	Polyethylene-imine	Molar ratio EG:EI	Structure of the block copolymer
56	CH_3O-PEG-NH_2 M_n ca. 2000	Ethyleneimine	1:1	AB diblock
57			1:2	AB diblock
58			1:10	AB diblock
59	CH_3O-PEG-NH_2 M_n ca. 5000	Ethyleneimine	1:1	AB diblock
60p			1:10	AB diblock
61	CH_3O-PEG-NH_2 M_n 10 000	Ethyleneimine	1:1	AB diblock
62			1:2	AB diblock
63			1:10	AB diblock
64	CH_3O-PEG-NH_2 M_n 20 000	Ethyleneimine	1:1	AB diblock
65			1:2	AB diblock
66			1:10	AB diblock

The polymers were characterized by ^1H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 56. They are representative of the other examples, for which very similar data were obtained.

^1H NMR (500 MHz, D_2O): δ/ppm = 2.60-3.00 (ethyleneimine CH_2), 3.78 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, D_2O): δ/ppm = 38.2 (ethyleneimine CH_2), 39.9 (ethyleneimine CH_2), 46.2 (ethyleneimine CH_2), 47.9

(ethyleneimine CH_2), 51.7 (ethyleneimine CH_2), 53.4 (ethyleneimine CH_2), 54.8 (ethyleneimine CH_2), 70.2 (ethylene glycol CH_2).

GPC (aminoethyl methacrylate gel, 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 21 000, M_w = 40 000, M_p = 16 000, PD = 1.9, monomodal.
Comparison with CH_3O-PEG-NH_2 (RAPP Polymere, 5000 Da): M_n = 9100, M_w = 14 000, M_p = 16 000, PD = 1.6, monomodal.

Example 67:

Preparation of a PEG-PEI copolymer (macroinitiator route)

Preparation of the macroinitiator

2 g (0.4 mmol, 1 eq.) of a monomethyl ether polyethylene glycol (Aldrich, MW 5000) are weighed into a 50 ml round-bottomed flask with magnetic stirring bar and reflux condenser and are dissolved in 25 ml of distilled chloroform. 0.31 g of tosyl chloride (1.6 mmol, 4 eq.) are added to the stirred polymer solution. Finally, 0.22 ml of triethylamine (0.16 g, 1.6 mmol, 4 eq.) are added to the mixture as catalyst. The mixture is heated under reflux for 18 h. To isolate and purify the polymer, the solution is poured into 500 ml of diethyl ether. The precipitated polymer is filtered off, washed with a large amount of diethyl ether and dried in vacuo. 1.90 g of a white, flaky substance are obtained (91% yield).

Preparation of the PEG-PEI block copolymer

0.5 g of the macroinitiator (0.096 mmol, 1 eq.) is weighed into a 25 ml round-bottomed flask with magnetic stirring bar and reflux condenser and is dissolved in 10 ml of distilled water. While stirring, 1 ml of ethyleneimine (0.832 g, 19.32 mmol, 200 eq.) is added dropwise, and the mixture is heated at 60°C for 24 h. The volatile components are removed under reduced pressure. A white, resinous substance remains and is redissolved in 10 ml of water and precipitated with 200 ml of tetrahydrofuran. The polymer is isolated by decantation and dried in vacuo. 0.95 g of a yellowish resinous substance is obtained (71% yield).

The following can be prepared in a similar way: (all monomethyl-PEGs are obtainable from Aldrich)

No.	Starting compounds			
	Polyethylene glycol	Polyethylene-imine	Molar ratio PEG:EI	Structure of the block copolymer
68	CH_3O-PEG-Ts M_n ca. 550	Ethyleneimine	1:10	AB diblock
69			1:50	AB diblock
70			1:200	AB diblock
71	CH_3O-PEG-Ts M_n ca. 750	Ethyleneimine	1:10	AB diblock
72			1:50	AB diblock
73			1:200	AB diblock

74	CH_3O-PEG-Ts M_n ca. 2000	Ethyleneimine	1:10	AB diblock
75			1:50	AB diblock
76			1:200	AB diblock
77	CH_3O-PEG-Ts M_n ca. 5000	Ethyleneimine	1:10	AB diblock
78			1:50	AB diblock

The polymers were characterized by ^1H and ^{13}C NMR spectroscopy and gel permeation chromatography. The following data were obtained for Example No. 67. They are representative of the other examples, for which very similar data were obtained.

^1H NMR (500 MHz, D_2O): δ/ppm = 2.80-3.20 (ethyleneimine CH_2), 3.80 (ethylene glycol CH_2).

^{13}C NMR (125 MHz, D_2O): δ/ppm = 37.9 (ethyleneimine CH_2), 39.4 (ethyleneimine CH_2), 46.1 (ethyleneimine CH_2), 47.2 (ethyleneimine CH_2), 51.3-52.7 (ethyleneimine CH_2), 70.2 (ethylene glycol CH_2).

GPC (aminoethyl methacrylate gel), 1% formic acid, 0.5 ml/min, 25°C, calibrated using pullulan standards): M_n = 35 000, M_w = 90 000, M_p = 52 000, PD = 2.6, monomodal.
Comparison with CH_3O-PEG-Ts 5000 Da): M_n = 4800, M_w = 7600, M_p = 8600, PD = 1.6, monomodal.

Abbreviations

bPEG	branched polyethylene glycol
bPEI	branched polyethyleneimine
CMC	critical micelle concentration
DMF	dimethylformamide
HMDI	hexamethylene diisocyanate
lPEG	linear polyethylene glycol
lPEI	linear polyethyleneimine
M_n	number average molecular weight
M_p	peak molecular weight
mPEG	monomethoxy polyethylene glycol
M_w	weight average molecular weight
MW	unspecified average molecular weight
PD	polydispersity
Ts	tosyl
σ_{min}	minimum surface tension

Biological Examples

I. Transfection experiments

The transfection properties of the polymers $PEI(PEG)_{20}$ (Example 1) and $PEG(PEI)_8$ (Example 27) were studied on the 3T3 cell line. 50 000 cells/well were seeded in 12 well plates and incubated for 24 hours (DMEM + 2 mM glutamine + 10% FCS, 37°C, 10% CO_2). The medium was then changed. 4 μg of pGL3 plasmid in 100 μl of 150 mM saline in each well were complexed with the appropriate amount of polymer in 100 μl of 150 mM saline and, after 10 minutes, added to the cells. After 4 hours, the medium was again changed and, after 48 hours, the evaluation took place. Luciferase expression was

determined using the Promega luciferase assay kit in a Berthold Sirius luminometer. The protein concentration was quantified with a modified BCA assay. The stated data are in each case the mean of three wells ± standard deviation for the corresponding nitrogen/phosphorus ratios.

Example 1: [PEI(PEG)$_{20}$]

Measured data:

N/P 5: 0.0057 ± 0.0036 ng/mg of protein

N/P 10: 0.1786 ± 0.1522 ng/mg of protein

N/P 20: 0.6952 ± 0.5498 ng/mg of protein

N/P 50: 5.1963 ± 2.6863 ng/mg of protein

(only plasmid: 0.0000 ± 0.00004 ng/mg of protein)

Example 27: [PEG(PEI)$_{8}$]

Measured data:

N/P 5: 0.0024 ± 0.0012 ng/mg of protein

N/P 10: 0.0045 ± 0.0046 ng/mg of protein

N/P 20: 0.0109 ± 0.0078 ng/mg of protein

N/P 50: 0.0765 ± 0.0498 ng/mg of protein

(only plasmid: 0.0000 ± 0.00004 ng/mg of protein)

In both cases it was possible to detect gene expression on the basis of transfection having taken place. Moreover, PEI(PEG)$_{20}$ shows a distinctly greater transfection efficiency than does PEG(PEI)$_{8}$.

II. In vitro cytotoxicity determination by the MTT assay:

The copolymers of Examples 1 and 27 were studied for their cytotoxicity in the cell culture model using the

MTT assay by the method of Mosmann (J. Immunol. Methods. 65: 55-63 (1983)). 8000 L929 mouse fibroblasts/well were preincubated in 96 wells for 24 h and treated with the polymer solutions at various concentrations for 3, 12 and 24 h. The mitochondrial activity was determined through the conversion of the MTT dye to the formazan, which was quantified by spectrophotometry. The polymers were employed as solutions in DMEM with 10% FCS in five different concentrations. If necessary, the pH was adjusted to 7.4 and the samples were sterilized by filtration (0.2 μm). The blends were prepared by mixing the two individual components (subtracting the amount of spacer). For the evaluation, the cellular viability [%] was plotted against the polymer concentrations employed, and the IC50 was determined.

Result:

- The in vitro cytotoxicity of the free polymers increases with increasing polymer concentration and with increasing incubation time.
- Copolymer of Example 1: The toxicity of the mixture of individual components PEI 25 kDa and PEG 550 Da corresponds to the toxicity of the free PEI 25 kDa. The tolerability is distinctly improved by the covalent linkage of the two components. Although the toxicity profile after 24 h corresponds to that of the individual components and thus to that of the free PEI 25 kDa, the cytotoxicity falls with shorter incubation periods. The PEG coating masks the positive charge of the polyethyleneimine, and thus the charge-mediated effects on cell membranes are

reduced.

- Copolymer of Example 27: The mixture of the two individual components PEI 700 Da and PEG 10 kDa showed no reduction in the viability of the cells up to 10 mg/ml. In the same concentration range, the copolymer showed an increased limitation on cellular viability after 3, 12 and 24 h, which can be explained by the increase in molecular weight.
- Example 27 shows less cytotoxicity than Example 1.

III. In vitro cytotoxicity determination by the LDH assay:

L929 mouse fibroblasts were seeded in the same cell density as in the MTT assay in 6-well multidishes, preincubated for 48 h and incubated with the polymer solution (in PBS pH 7.4) for 1, 2, 3 and 6 h. The extracellular LDH fraction was quantified with a standard kit (Sigma, DG-1340-K) by photometric determination of the reduction of NAD in the presence of lactate and LDH. To determine the 100% value, cells were lyzed with 0.1% Triton X-100.

Result:

The LDH assay confirms the results of the MTT test. Correlation of the two assays shows that membrane damage starts first and, after a time lag, the reduction in metabolic activity starts. The membrane-damaging effect of the polymers becomes stronger as the incubation time and polymer concentration increase.

IV. DNA binding of the copolymers determined by agarose gel electrophoresis

The binding capacity of the copolymers of Examples 1 and 27 was determined by electrophoresis on 1% agarose gels at 80 V. The plasmids (CMV-nlacZ) are located by UV excitation at 254 nm after ethidium bromide staining.

Result:

- Both polymers are capable of electrostatic interaction with the plasmid.
- Consistent with the blend, the polymer of Example 1 is able to bind plasmid completely from a nitrogen-PEI/phosphate-DNA ratio (N/P ratio) of 1.7 onwards. The ethidium bromide exclusion observed with the blend (from N/P 5.8), a sign of intensive DNA condensation, is incomplete for the copolymer up to N/P 23.0.
- Whereas for the blend of Example 27 complete plasmid binding is to be observed only from N/P 4.1 onwards, and no complete ethidium exclusion is to be observed, the copolymer showed plasmid binding from N/P 2.4 onwards and exclusion of the dye from N/P 16.6 onwards.

V. Erythrocyte aggregation assay

Erythrocytes were isolated from the citrated blood of Wistar rats by the method of Parnham and Wetzig (Chem. Phys. Lipids, 1993, 64: 263-274), seeded in 24 wells and incubated with the test solutions at 37°C for 2 h. The aggregation and adhesion of the erythrocytes under the influence of the polymer were examined under the microscope. Untreated erythrocytes served as control.

Result:

- Free copolymer of Example 1 showed at concentrations of 0.27-18 μg/well by comparison with the blend and with PEI 25 kDa a reduced aggregation and adhesion of the red blood corpuscles to the cell culture dishes. Whereas no significant differences were to be seen at low concentrations (0.27-0.7 μg/well), a marked difference between copolymer and blend or PEI 25 kDa was detectable with increasing concentration. The aggregating effect increases as the N/P ratio increases.
- Copolymer of Example 27 showed the opposite behavior. Aggregation of the blend and of free PEI is less pronounced than that of the copolymer.
- The erythrocyte aggregation is significantly reduced through complexation of both copolymers with plasmid DNA compared with the free polymer.

VI. Hemolysis assay

Erythrocytes were isolated from the citrated blood of Wistar rats by the method of Parnham and Wetzig (Chem. Phys. Lipids, 1993, 64: 263-274), mixed with the polymer solutions and incubated at 37°C for 1 h. The erythrocytes are pelleted by centrifugation (10 min, 25°C, 700 g), and the hemolyzate is measured by photometry on the supernatant at 540 nm.

Result:

- The individual components PEG 8-arm, PEG 500 Da and PEI 700 Da show no significant hemolytic effects in the concentration range 0.001-10 mg/ml

(all 1-3%).

- The copolymer of Example 27 likewise shows no pronounced effects (<5%) in the same concentration range.
- With the individual components PEI 25 kDa and with the blend for Example 1, the hemolytic activity increases at 0.001-10 mg/ml (22.13% at 10 mg/ml).
- The copolymer of Example 1 shows an increasing lytic activity of up to 13.30% up to 0.5 mg/ml, while the hemolytic effect decreases again at higher concentrations up to 10 mg/ml (2.90% at 10 mg/ml).

VI. Pharmacokinetics and organ distribution of polymer-DNA complexes in mice

The pharmacokinetics and organ distribution of the copolymers of Example 1 and 27 were determined in balb/c mice. The polymers were radiolabeled with ^{125}I Bolton Hunter reagent (Pharmacia Biotech). Amounts of 0.4 or 0.04 or 0.008 mg of PEI (component) per kg of mouse were complexed with the appropriate amount of NF-κB decoy oligodeoxynucleotide (ODN) in the nitrogen/phosphorus ratio N/P 3.5 or N/P 6 in a total volume of 80 μl in 5% glucose solution and, after 10 minutes, injected into the anesthetized mice via the subclavian vein. After 20 seconds, 1, 2, 5, 15, 30, 60, 90 and 120 minutes, blood samples were taken from the arteria aorta communis through a catheter. The urine was collected through a bladder catheter for 120 minutes. After 120 minutes, the mice were decapitated and the organs cortex, kidney, liver, heart, lung, spleen and adipose tissue were removed.

The amount of polymer in the samples was determined by measuring the radioactivity with a 1277 Gammamaster automatic gamma counter (LKB Wallac).

The data were analyzed using the Kinetica 1.1 program and a 2-compartment model for i.v. bolus injection. The volume of distribution (V_c), the elimination constant (k_{el}) and AUC were calculated from the blood level plots. Mean ± standard deviation are stated when three animals could be analyzed, the median is stated for two animals, and the value is stated in parentheses when there was only one animal.

Complex preparation and dosages

Polymer	N/P	Dose [mg/kg]	V_c [ml]	k_{el} [min^{-1}]	AUC [min µg ml^{-1}]
25 kDa PEI	3.5:1	0.4	23.39	0.106	4.89
Example 1	3.5:1	0.4	(4.54)	(0.028)	(79.03)
Example 27	3.5:1	0.4	5.84±0.4	0.104±0.017	16.86±1.64
25 kDa PEI	6:1	0.4	5.39	0.099	19.22
25 kDa PEI	6:1	0.04	1.37±0.2	0.14±0.026	6.22±1.18
25 kDa PEI	6:1	0.008	9.57±1.78	0.063±0.009	0.34±0.1
Example 1	6:1	0.4	6.20	0.067	27.84

Example 1	6:1	0.04	3.37±0.32	0.072±0.01	4.0±0.67
Example 1	6:1	0.008	5.1±0.55	0.054±0.004	0.80±0.10
Example 27	6:1	0.4	8.12	0.0593	21.72

Result:

- Observations with a relatively low dose indicate that the toxicity of PEI(PEG)$_{20}$ is weaker than that of PEI 25 kDa.
- The plasma levels of all the polymers could be described by a 2-compartment model.
- The copolymers have a higher AUC and a smaller volume of distribution than the 25 kDA PEI. PEI(PEG)$_{20}$ (Example 1) has a larger effect than PEG(PEI)$_8$ (Example 27).
- Elimination was reduced with the copolymers.
- V_c and k_{el} show no detectable dose-dependency.
- The calculated AUC for PEI 25 kDa and Example 1 was proportional to the dose, while the gradient of the AUC/dose lines was larger with the copolymer of Example 1.
- The main organs of distribution after 120 minutes were liver, kidney and spleen. For the 6:1 complexes, the copolymers show a reduced uptake in liver and spleen and a higher uptake in the kidney compared with PEI 25 kDa.

New Claims

1. A compound of the formula I or II

(I) $A(-X-B)_n$ (II) $C(-Y-D)_m$

in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 100 to 10 000 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 100 to 1 000 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 1 to 20,

-OC(O)NH(aryl)NHC(O)- with aryl = aromatic unit,

$-O(CH_2)_pC(O)-$ with p = 1 to 10,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 20;

n is an integer from 1 to 200;

C is a linear or branched PEI with a molecular weight of from 100 to 1 000 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n′ is from 3 to 25 000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit,

$-(CH_2)_tC(O)O-$ with t = 2 to 10,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 20,

and

m is an integer from 1 to 200,

with the proviso that the radicals and variables in formula II are defined so that no compounds of

the formula I are included thereby.

2. A compound as claimed in claim 1, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 1000 to 100 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 100 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 2 to 10,

$-OC(O)NH(aryl)NHC(O)-$ with aryl = aromatic unit with one nucleus,

$-O(CH_2)_pC(O)-$ with p = 1 to 3,

$-OCH_2CH(OH)CH_2-$,

$-OC(O)-$, or

$-O(CH_2)_q-$ with q = 1 to 6,

n is an integer from 1 to 50,

C is a linear or branched PEI with a molecular weight of from 400 to 100 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n′ is from 10 to 5000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit with one nucleus,

$-(CH_2)_tC(O)O-$ with t = 2 to 3,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 6;

and

m is an integer from 1 to 100,

with the proviso that the radicals and variables

in formula II are defined so that no compounds of the formula I are included thereby.

3. A compound as claimed in claim 1 or 2, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 5000 to 50 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 50 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 4 to 6,

$-OC(O)NH(aryl)NHC(O)-$ with aryl = tolyl,

$-O(CH_2)_pC(O)-$ with p = 1,

$-OCH_2CH(OH)CH_2-$,

$-OC(O)-$, or

$-O(CH_2)_q-$ with q = 1 to 3;

n is an integer from 1 to 12;

C is a linear or branched PEI with a molecular weight of from 400 to 50 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 10 to 1000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

-C(O)NH(aryl)NHC(O)O- with aryl = tolyl,

$-(CH_2)_tC(O)O-$ with t = 2,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 3;

and

m is an integer from 1 to 50,

with the proviso that the radicals and variables in formula II are defined so that no compounds of

the formula I are included thereby.

4. A compound as claimed in any of claims 1 to 3, which has formula I.

5. A compound as claimed in any of claims 1 to 3, which has formula II.

6. A compound as claimed in any of claims 1 to 4, in which X is a linker of the formula $-OC(O)NH(CH_2)_oNHC(O)-$.

7. The use of a compound of the formula II in which Y is a linker of the formula $-C(O)NH(CH_2)_sNHC(O)O-$ with s = 1-10, and the other radicals are as defined in any of claims 1 to 3, for the complexation of polynucleic acids in aqueous systems.

8. A process for preparing a compound of the formula I as claimed in any of claims 1 to 4, which comprises

a) reacting compounds of the general formula V

(V) $A-(OH)_n$ with A and n = as in formula I

with diisocyanate, or

b) adding compounds of the general formula VI

(VI) $A-(NH_2)_n$ (with A and n = as defined in

formula I)

to the reaction mixture for the polymerization of ethyleneimine before the start of the polymerization or not until the polymerization is in progress, or

c) employing compounds of the general formula VII

(VII) $A-(OS(O)_2R^4)_n$ with A as in formula I and R^4 = aliphatic or aromatic radical as macroinitiator for the polymerization of ethyleneimine.

9. A process for preparing compounds of the formula II as claimed in any of claims 1 to 3 and 5, which comprises initially reacting compounds of the general formula IX

(IX) D-OH (with D as defined in formula II)

with diisocyanate and subsequently reacting the resulting compound with linear or branched polyethyleneimine.

(VIII)

10. The use of a compound of the formula I or II

(I) $A(-X-B)_n$ (II) $C(-Y-D)_m$

in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 100 to 10 000 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 100 to 1 000 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 1 to 20,

-OC(O)NH(aryl)NHC(O)- with aryl = aromatic unit,

$-O(CH_2)_pC(O)-$ with p = 1 to 10,

-OC(O)-, or

$-O(CH_2)_q-$ with q = 1 to 20;

n is an integer from 1 to 200;

C is a linear or branched PEI with a molecular weight of from 100 to 1 000 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n′ is from 3 to 25 000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal is linked to a nitrogen atom of the PEI:

$-C(O)NH(CH_2)_sNHC(O)O-$ with s = 1 to 20,

$-C(O)NH(aryl)NHC(O)O-$ with aryl = aromatic unit,

$-(CH_2)_tC(O)O-$ with t = 2 to 10,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 20,

and

m is an integer from 1 to 200,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby,

for the complexation of polynucleic acids in aqueous systems.

11. The use as claimed in claim 10, wherein a compound of the formula I or II, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 1000 to 100 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 100 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 2 to 10,

$-OC(O)NH(aryl)NHC(O)-$ with aryl = aromatic unit with one nucleus,

$-O(CH_2)_pC(O)-$ with p = 1 to 3,

$-OCH_2CH(OH)CH_2-$,

$-OC(O)-$, or

$-O(CH_2)_q-$ with q = 1 to 6,

n is an integer from 1 to 50,

C is a linear or branched PEI with a molecular

weight of from 400 to 100 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$-(CH_2CH_2O)_{n'}-R^1$

which is bonded via O and in which n' is from 10 to 5000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-C(O)NH(CH_2)_sNHC(O)O-$ with s = 2 to 10,

-C(O)NH(aryl)NHC(O)O- with aryl = aromatic unit with one nucleus,

$-(CH_2)_tC(O)O-$ with t = 2 to 3,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 6;

and

m is an integer from 1 to 100,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby,

is used.

12. The use as claimed in either of claims 10 or 11, wherein a compound of the formula I or II, in which

A is a hydrophilic, nonionic, linear or branched polymer with a molecular weight of from 5000 to 50 000 g/mol;

B is a linear or branched polyethyleneimine (PEI) with a molecular weight of from 400 to 50 000 g/mol;

X is a direct linkage of blocks A and B or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-OC(O)NH(CH_2)_oNHC(O)-$ with o = 4 to 6,

$-OC(O)NH(aryl)NHC(O)-$ with aryl = tolyl,

$-O(CH_2)_pC(O)-$ with p = 1,

$-OCH_2CH(OH)CH_2-$,

$-OC(O)-$, or

$-O(CH_2)_q$ with q = 1 to 3;

n is an integer from 1 to 12;

C is a linear or branched PEI with a molecular weight of from 400 to 50 000 g/mol;

D is a residue of a polyethylene glycol of the formula

$$-(CH_2CH_2O)_{n'}-R^1$$

which is bonded via O and in which n' is from 10 to 1000, and R^1 is hydrogen, an aliphatic radical or another OH-protective group or a cellular ligand;

Y is a direct linkage of blocks C and D or a linker with the following structures whose C-terminal side is linked to a nitrogen atom of the PEI:

$-C(O)NH(CH_2)_sNHC(O)O-$ with s = 4 to 6,

$-C(O)NH(aryl)NHC(O)O-$ with aryl = tolyl,

$-(CH_2)_tC(O)O-$ with t = 2,

$-CH_2CH(OH)CH_2O-$, or

$-(CH_2)_uO-$ with u = 1 to 3;

and

m is an integer from 1 to 50,

with the proviso that the radicals and variables in formula II are defined so that no compounds of the formula I are included thereby,

is used.

13. The use of a compound as claimed in any of claims 10 to 12 for the complexation of DNA in aqueous systems.

14. The use of a compound as claimed in any of claims 10 to 12 for the complexation of RNA in aqueous systems.

15. The use of a compound as claimed in any of claims 10 to 12 for the complexation of ribozymes in aqueous systems.

16. A composition which comprises at least one nucleic acid and one compound of the formula I or II which is as defined in any of claims 10 to 12.

17. The use of a compound of the formula I or II which are as defined in any of claims 10 to 12 as surfactant.

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Cationic Block Copolymers

the specification of which is attached hereto unless the following box is checked:

☒ was filed on January 11, 2002 as United States Application Number 10/030,803 or PCT International Application Number .... and was amended on ....(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 (a)-(d) or § 365 (b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT International Application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	MONTH/DAY/YEAR FILED	PRIORITY CLAIMED
199 33 024.7	*Federal Republic of Germany*	*July 15, 1999*	*YES*

I hereby claim the benefit under Title 35, United States Code §119 (e) of any United States provisional application(s) listed below:

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365 (c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS: PATENTED, PENDING, ABANDONED
PCT/EP00/06214	July 4, 2000	Pending

I hereby appoint as my attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Gregory N. Clements, registration no. 30,713, as well as Klaus Schweitzer with Limited Recognition under 37 C.F.R. §10.9(b).

Address all correspondence to **ProPat, L.L.C., Crosby Building, 2912 Crosby Road, Charlotte, North Carolina 28211-2815, Attention: Klaus Schweitzer**. Address telephone communications to **Klaus Schweitzer** at **(704) 365-4881.**

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

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